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# ADVANCES IN CARBOHYDRATE CHEMISTRY

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VOLUME 2



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## EDITORS' PREFACE

The first volume of this series was published during the war and contained chapters written only by American authors. In this second volume we are especially pleased to be able to present several articles from the English school of carbohydrate chemists and one from France, thus making the "Advances" international in scope. Dr. Stanley Peat of Birmingham University, England, has been of great help in making arrangements for these and future articles. As communications between countries become facilitated, we hope to increase the number of countries that the participating authors represent.

We wish again to extend a cordial invitation to carbohydrate chemists to suggest topics in need of review and to suggest any way in which our contributions to the field of carbohydrates may be improved.

Dr. L. T. Capell has again compiled the subject index. The editorial assistance rendered by Edgar E. Dickey and Mary Grace Blair has been greatly appreciated. Dr. Claude S. Hudson has given invaluable aid in the editing of this and the preceding volume.

The journal abbreviations used are those employed by Chemical Abstracts. Unless otherwise noted, all temperature values are expressed in centigrade units.

Appleton, Wisconsin  
Columbus, Ohio

THE EDITORS  
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# MELEZITOSE AND TURANOSE

By C. S. HUDSON

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## INTRODUCTION

The modern scientific record concerning melezitose begins in the year 1822, and it originates in France. The growth of our information regarding this sugar has revealed so extensive an occurrence of it in nature that it is no longer a scientific curiosity, as it was in earlier years. It has been found in the exudations from so many plants of widely different botanical classifications that it should be recognized as one of the more important and widely distributed sugars of nature. In some localities such plant exudations are collected by bees when floral nectar is scarce; in consequence, melezitose is often found in so-called "honeydew" honey, which can become a firm magma as a result of the crystallization of this sugar. In recent years the professional apiarists in some localities of the Eastern United States have become quite familiar with the recondite name of this sugar; to them "melezitose honey" means trouble, because the sugar crystallizes so rapidly in the comb that it interferes with their centrifuging process for making "extracted" liquid honey. It is difficult to believe that prehistoric man was not acquainted with crystalline melezitose in places where it is often abundant; he did not need to invent the art of crystallizing it, as was the case with sucrose.<sup>1</sup> In some localities of British Columbia the bears know melezitose; surely early man of that section did not overlook it. In a vast desert region of Asia an exudation from a low bush named "camel's thorn" (*Alhagi camelorum* or *maurorum*) is known to have been an article of extensive commerce for at least a thousand years; this dried "manna" sometimes contains much crystalline melezitose. Probably the only crystalline sugars that were known to prehistoric man were melezitose and D-glucose, the latter often being present in crystallized honey and on the surface of raisins and some other dried fruits.

## I. THE LARCH MANNA OF BRIANÇON

A memoir was published in 1822 by Moringlane, Duponchel and Bonastre<sup>2</sup> in response to a question which had been proposed by the Paris Society of Pharmacy for investigation, the subject of the proposal being "to establish a precise distinction among the diverse natural products of the *Terebinthaceae* and the *Coniferae*." The twenty-page memoir is devoted principally to the description of the characteristic

(1) E. O. Von Lippmann, "Geschichte des Zuckers," Max Hesse's Verlag, Leipzig (1890). The art of crystallizing sucrose seems to have had its crude beginning in India at some period between 300 and 600 A.D., probably nearer the later date, but sirups from the juices of sugar canes were doubtless made in far earlier times.

(2) Moringlane, Duponchel and Bonastre, *J. Pharm.*, [2] 8, 329 (1822).

properties of the turpentine and resins of commerce, but in the section relating to the products from the larch tree (*Larix europaea* DC.), the authors describe, in addition to Venice turpentine, two substances which are soluble in water and therefore are not of a resinous nature. One of these is a gum which was known in Russian pharmacies as Orembourg gum and was considered to resemble gum arabic; the other is a kind of manna that was found at times on larch trees in France and was commonly known as "manna of Briançon," from the locality where it had become recognized. They describe the manna as follows (in translation):

"It is a secretion which forms on the young larch trees, or better even upon the older ones where it is present on the twigs of the new growth. The secretion is the more abundant if the summer is dry and hot, cold winds being unfavorable for it. It is collected in the very early morning when there is still dew, because it disappears completely when the sun has risen. There are years when it is not purchasable even at an offering of twenty-four francs an ounce, but during severe droughts it is obtainable from the children of the shepherds. Occasionally some branches of a larch tree are so charged with the manna that they appear as though snow had fallen upon them. The secretion consists of small sticky whitish grains, of dull sweetish flavor; the shepherds find them to be purgative but this property is less active than in the case of the manna of the ash tree."

Eleven years later the inquiring pharmacist Bonastre<sup>3</sup> resumed the study of the sample of Briançon manna which he had procured in 1822, and he now says that the manna is not an article of commerce but that (in translation) "nevertheless, as an object of natural history, of pharmaceutical and medical interest, it is not to be disdained. Its formation on a tree of the family of the conifers, which furnishes turpentine in abundance, its prompt appearance at some period which is not fully predictable, and its sudden disappearance under certain solar and atmospheric influences, naturally make it a subject worthy of study." He determined that the principal component of Briançon manna is a substance which may be recrystallized from water and is different from the well known mannitol [designated D-mannitol later by Emil Fischer] of Calabrian manna, the manna of the ash tree (*Fraxinus Ornus*, L.). He describes the crystals from the Briançon manna as growing from concentrated aqueous solution in the form of "square platelets arranged about a common center, widening as they diverge."

Twenty-three years elapsed after the studies of Bonastre before this

(3) Bonastre, *J. Pharm.*, [2] 19, 443, 626 (1833).

curious manna of Briançon attracted attention again. Berthelot,<sup>4</sup> in 1856, obtained a small sample of it (two grams), with which he was able to make a preliminary study of its crystalline component that Bonastre had described. This pure crystalline substance seemed to be a non-reducing compound sugar, as shown by its analysis and its reduction of Fehling solution after acid hydrolysis. It was difficultly fermentable by yeast but fermented readily after hydrolysis. It resembled sucrose but differed by its crystal form and its much larger rotation. Three years later Berthelot succeeded in obtaining a sufficient quantity of Briançon manna to enable him to characterize the saccharine component more specifically; on the basis of these data he definitely classified the pure crystalline substance as a new compound sugar, to which he gave the name melezitose (French *le mélèze*, the larch tree<sup>5</sup>). The sugar contained somewhat more than 4% water of crystallization, the exact proportion being uncertain because of efflorescence. After its acid hydrolysis, D-glucose was identified. The crystallization of melezitose in square plates, which Bonastre had mentioned, was confirmed; its specific rotation, as anhydrous melezitose, for the "teinte de passage" was found to be  $[\alpha]_D +94.1^\circ$  in water, a value which corresponds to about  $[\alpha]_D +88.4^\circ$ .

## II. THE ALHAGI MANNA (TURANJABINE)

In 1870, Messrs. Allen and Hanburys, pharmacists of London, presented to Berthelot a sample of a manna which had been sent from Lahore by Dr. Burton Brown; it was described as an exudation from a spiny bush, *Alhagi maurorum*, belonging to the *Leguminosae*. This manna was said to be very abundant in Persia, where it was used as a purgative and even as a food under the name "taranjbin." The manna was well known to Avicenna and other writers of the Middle Ages; references to them may be found in Von Lippmann's "Geschichte des Zuckers,"

(4) M. Berthelot, *Ann. chim. phys.*, [3] 46, 66 (1856); 55, 282 (1859). He mentions that the former use of the manna in pharmacy had ceased and that it was not an article of commerce in 1859. Arnhart (ref. 22) records that the Paris tariff regulations of the year 1542 mention the *manna Brianzona* or *Briantica*. Nicholas Lemery's *Dictionnaire des Drogues*, third edition, p. 298 (Amsterdam, 1716) carries the following statement: "Il découle des grosses branches des Melezes qui croissent en grande quantité dans le haut Dauphiné, principalement aux environs de Briançon, une manne blanche et seche qu'on appelle en Latin *Manna laricea* et en Francois *Manne de Briançon*. Elle est purgative."

(5) The Latin classical name of the tree was *larix*, but the authorities have not found any historical relationship of the French to the Latin name; they classify *mélèze* as originating in Alpine patois. The word *larix* occurs many times in Pliny's *Natural History*.

page 83,<sup>1</sup> and additional ones in the article of reference 25. Berthelot commissioned his student Villiers to study the saccharine material contained in the manna. Villiers<sup>6</sup> found that melezitose was the principal component; the pure recrystallized sugar was observed to be a hydrate containing 5.3% water, and its specific rotation, on the anhydrous basis, was  $[\alpha]_D +94.6^\circ$  and  $[\alpha]_D +88.9^\circ$ . Measurements of angles of the crystals were recorded. In a note appended to Villiers' article, Berthelot calls attention to the probability that melezitose is widely distributed in nature, since it had been found in exudations from a conifer and a legume and in widely separated regions.

A record<sup>7</sup> that is of some historical interest discloses that the French Academy of Sciences had received a sample of this Persian or Alhagi manna from De Mirbel in 1836 and from Ravergin in 1830.

Seven years after the termination of the researches of Berthelot and Villiers on melezitose, V. Markovnikoff, professor in the university of Moscow, began a study of the manna from the Alhagi plant. The following full quotation has been translated by Dr. Elias Yanovsky from the *Journal of the Russian Physico-chemical Society*, 16 (chemical section), 330 (1884).

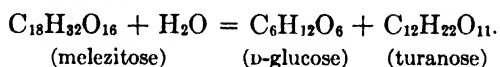
"V. Markovnikoff Reports on Turkestan Manna. A considerable amount of manna that is eliminated by the herbaceous plant *Alhagi maurorum* or *camelorum* is collected in some localities of the Turkestan region. Purified manna partly replaces sugar for the natives. Raw manna that was obtained by V. Markovnikoff, through the cooperation of Mrs. Fedchenko, contained many pods and other small parts of the plant held together by a soft saccharine material. The native name for the manna is "tarandjabin." At first, the method of purification consisted in extraction with water, evaporation to dryness, and extraction with alcohol, which dissolves the non-crystalline part easily but the crystalline sugar only with difficulty; however, during the final evaporation of the water extract part of the crystalline sugar apparently changes to non-crystalline, and therefore it is better to bring the water extract to the point of crystallization by careful evaporation at ordinary temperature. By recrystallization from water, perfectly pure and transparent crystals are obtained. This saccharine material has a weak sweet taste; it is less soluble in water than cane sugar and practically insoluble in concentrated alcohol. Water solutions mold very quickly. The properties and analytical data indicate that the substance is a sugar. It does not reduce Fehling's solution even on heating. On heating with

(6) A. Villiers, *Ann. chim. phys.*, [5] 12, 433 (1877).

(7) *Compt. rend.*, 3, 479 (1836).

dilute sulfuric acid, or even weak organic acids, decomposition into non-crystalline glucose takes place, which very strongly reduces copper salts. On closer investigation this sugar proved to be identical with melezitose which was investigated by Berthelot, rather superficially due to the lack of material. The crystals contain one molecule of water,  $C_{12}H_{22}O_{11} + H_2O$ , which they lose at  $100^\circ$ , and then melt at  $140^\circ$ . The specific rotation,  $[\alpha]_D$ , is  $+88.07^\circ$  according to a determination by A. Alekhin, who undertook the detailed investigation of the components of this manna. According to all these data the Turkestan manna appears to be identical with the Persian manna investigated by Villiers in 1877. Secretary of the Committee, A. Alekhin."

During the early stage of Alekhine's research<sup>8</sup> he accepted the view of Berthelot and Villiers that melezitose is a disaccharide having the formula  $C_{12}H_{22}O_{11} \cdot H_2O$ ; its water content, which was found to be approximately 5%, agreed with this formula. His important discovery that melezitose is really a trisaccharide was the result of his following the course of its acid hydrolysis through polariscopic readings. A solution of melezitose in 1% sulfuric acid on the steam bath changed in  $[\alpha]_D$  value from  $+87.5^\circ$  to  $+64^\circ$  in thirty minutes and remained nearly constant during the following half hour ( $+63.8^\circ$ ); thereafter the rotation decreased very slowly and seemed to become constant at about  $+50^\circ$ , the solution having become much colored, however, through decomposition. He concluded that the hydrolysis proceeded in two stages of greatly different speeds; the first rate was comparable with the rapid speed for sucrose and the second with the slow speed for maltose. Such behavior excluded the possibility that melezitose could be a *disaccharide*; it required that the sugar be at least a *trisaccharide*. A molecular weight determination by the new freezing-point depression method of Raoult showed the value for a trisaccharide, and Alekhine thus established the equation for the first stage of the acid hydrolysis of melezitose:



The disaccharide component was not maltose; the value of the rotation at the end of the first stage ( $+64^\circ$ ) excluded that sugar. The fairly pure, though amorphous, disaccharide, which he freed largely from D-glucose by the use of alcohol, was considered to be a new disaccharide, to which Alekhine gave the name *turanose*, from *Turan*, the old Persian

(8) A. Alekhine, *Ann. chim. phys.*, [6] 18, 532 (1889). See also *Bull. soc. chim.*, 46, 824 (1886).

name (mentioned in the Avesta) of the general region, northeast of Persia, from which the manna had come.<sup>9</sup>

Alekhine discovered the fully acetylated crystalline derivative of melezitose, the hendecaacetate, which has become of aid in the precise identification of melezitose. It crystallizes readily; m. p. 117°,  $[\alpha]_D^{20} +111^\circ$  in benzene and  $+104^\circ$  in chloroform (ref. 17).

The following is a translation of parts of Alekhine's description of the origin of Alhagi manna; one may conclude from it that this manna will probably always be a good source for supplies of the trisaccharide.

"Persian Manna or Terenjabine. Terenjabine is common only in the oriental countries. In the Middle Ages, it was imported into Europe under the name fruit-honey (*miel de fruits*). It is mentioned by Avicenna and Serapion under the name "terenjabine." Persian manna is a secretion of the *Alhagi maurorum* DC. (*syn. Hedyarum alhagi mannifera* L.), a spiny bush belonging to the *Papilionaceae*. It is a very common plant in the deserts of the Orient and also in the Kirghis steppes of Russia; but the exudation of the manna is noticed only in certain countries such as Bokhara, Persia, Arabia, and Palestine. In addition to the name "terenjabine," which is an Arabian word modified through the Persian, the manna is known in Tashkend as "*rousta*," whereas the manniferous plant is called "*yantak*," from which is derived the other name of the manna, "*yantak-chakar*," meaning *yantak sugar*. The season for collection of the manna lasts three or four weeks in June during the period when the seeds mature. The method of collection is simple; a cloth is spread around the bush and the manna is dislodged by striking the branches with a rod. The collecting is done in the early morning and if the yield is good a worker can harvest about 25 kg. In the market at Tashkend three or four grades of "*rousta*" are sold at from 4 to 8 rubles per poud, according to the quality of the product. The trade is considerable, since at Tashkend alone there are sold about 1000 pouds a year." (Reckoning the ruble at fifty cents and the poud as 16 kg., the annual sale at Tashkend was thus about 16,000 kg. at a price of 12 to 25 cents each, in 1889.) Alekhine states that "the presence of insects on the Alhagi plant, to the action of which the manna might be attributed, was not noticed at the time of collection."

There is a numerical error of importance in Alekhine's memoir which escaped notice until it was detected by Georges Tanret seventeen

(9) The adjective *turanian*, sometimes used by philologists to designate a certain group of related languages, is of the same origin. Possibly the preferred spelling in English of the mixed Persian and Arabic name of the Alhagi manna would be *turanjabine*, signifying *manna of Turan*. Many variations of the name are found in the scientific literature (*turanjin*, *tarondjabin*, *terendjabine*, *turjanbin*, etc.).

years later. The hydrated form in which melezitose crystallizes from water was found by Villiers and by Alekhine to contain slightly more than 5% water of hydration. Villiers regarded the sugar as a monohydrated disaccharide,  $C_{12}H_{22}O_{11} \cdot H_2O$ , this formula requiring 5%. Alekhine wrote the formula for the trisaccharide as  $C_{18}H_{32}O_{16} \cdot 2H_2O$ , stating that this formula also corresponds to 5%. However, his formula really corresponds to 6.67%. For a hydrated trisaccharide showing 5% water of hydration the formula that was disclosed by Alekhine's work should have been written  $C_{18}H_{32}O_{16} \cdot 1\frac{1}{2}H_2O$ . Later, as will be described, the correct formula, which is indeed  $C_{18}H_{32}O_{16} \cdot 2H_2O$ , was established by Georges Tanret.

### III. THE PRESENCE OF MELEZITOSE IN SEVERAL TYPES OF HONEYDEW

Four years after the publication of Alekhine's article, Maquenne<sup>10</sup> discovered the presence of melezitose in honeydew, a substance which he describes as follows (in translation): "It is well known that during dry years the leaves of certain trees become covered with a sticky exudation, sufficiently sweet to attract insects, and at times so abundant that sirupy droplets form and fall to the ground. This abnormal secretion is especially noticeable on linden and maple leaves, which then appear as though varnished; it has received the name *miellée* or *miellat* [English, honeydew], because of its flavor." He then mentions the rival views that the secretion results from the action of insects, particularly aphids, or that it is a true secretion from the leaves; decision is reserved, but Maquenne adds that he was not able to find insects in a batch of 100 kg. of linden leaves which carried 500 g. of honeydew. He was able to obtain crystalline melezitose in high yield, about 40%, from this honeydew of the linden (*Tilia*, species not stated). In concluding his memoir, he proposes the question whether melezitose may be a constituent of the normal tissues of those plants that yield secretions in which it is found. This fundamental question, which was advanced by Maquenne, has never received a decisive answer by experiment up to the present time. The species of the trees from which Maquenne collected the linden leaves is not stated, but the possibilities seem limited to *T. platyphyllos* Scop., *vulgaris* Hayne and *cordata* Mill. The flowers of two species of linden (*T. tomentosa* Moench and *petiolaris* Hook.) are said to be poisonous to bees.<sup>11</sup>

(10) L. Maquenne, *Bull. soc. chim.*, [3] 9, 723 (1893).

(11) Alfred Rehder, in L. H. Bailey's "Standard Cyclopedia of Horticulture," p. 3345, Macmillan (1930).

In later years honeydews from several other sources have been found to contain melezitose. Georges Tanret<sup>12</sup> isolated 22 g. of the crystalline sugar from a sirup containing 55 g. total solids which was obtained by washing 6 kg. of poplar leaves (*Populus nigra* L.). He also found melezitose in a sample of honeydew that had been collected by Boudier<sup>13</sup> from the "golden-chain" tree of South Europe (*Cytisus laburnum* L., presumably *Laburnum anagyroides* Griseb.). Boudier describes his field study of the production of honeydew by aphids; they attach themselves at the veins on the lower side of the leaves, draw in sap and expel from their abdominal extremity fine droplets in mist form which settle and dry on the upper surfaces of lower leaves. Von Lippmann<sup>14</sup> found melezitose in a sample of honeydew from bamboo (*Bambusa*). The honeydews which insects form from the larch tree and from *Pinus virginiana* contain melezitose, as will be described later. If it should be found on careful examination that the Alhagi manna results from the action of insects, it would also be classed as a honeydew.

#### IV. TURANOSE PHENYLOSAZONE

Alekhine had mentioned that turanose reacts with phenylhydrazine. Maquenne prepared the phenylosazone and described its precipitation from water in the flocculent or gel condition that is so very characteristic of the substance, but he did not report an analysis. A year after Maquenne's publication, Emil Fischer<sup>15</sup> examined a small sample of amorphous turanose which had been sent by Dr. Konowaloff of Moscow. Its phenylosazone was prepared in good crystalline form by several recrystallizations from aqueous alcohol; the analysis of these crystals proved conclusively the disaccharide formula for turanose which Alekhine had proposed from the analysis of amorphous substances. The writer had occasion recently to prepare turanose phenylosazone in considerable quantity; its very characteristic properties and those of the derived phenylosotriazole are described on pages 27 and 28.

#### V. ESTABLISHMENT OF THE TRUE COMPOSITION OF MELEZITOSE AND TURANOSE

Twelve years after Fischer's study of turanose phenylosazone there appeared a memoir by Georges Tanret<sup>16</sup> which has become the foundation

(12) G. Tanret, *Bull. soc. chim.*, [4] 27, 56 (1920).

(13) Boudier, *Association française pour l'avancement des sciences, Congrès de Blois*, (1884), *second part*, p. 289. Copy in the Library of Congress, Washington.

(14) E. O. Von Lippmann, *Ber.*, 60, 163 (1927). He remarks that the relationship between honeydew and aphids was noticed by Leeuwenhoek (1632-1723).

(15) E. Fischer, *Ber.*, 27, 2486 (1894).

(16) G. Tanret, *Bull. soc. chim.*, [3] 35, 816 (1906).



for all later researches on melezitose and turanose. Previous to his work it had been believed that these sugars were composed solely of D-glucose units, because only this monose had been detected in the products from their acid hydrolysis. Alekhine had detected D-glucose and turanose as the products from the first stage of the acid hydrolysis of melezitose, but he identified only D-glucose from the hydrolysis of turanose and therefore considered the disaccharide to be a D-glucosyl-D-glucose, a close relative of maltose. Tanret proved that turanose is composed of D-glucose and D-fructose by isolating the two monoses in crystalline form and in about equal proportions, from the acid hydrolysis of turanose *under conditions which did not decompose the fructose extensively*. The two monoses could come from either a D-glucosyl-D-fructose or a D-fructosyl-D-glucose; he proved that turanose is a D-glucosyl-D-fructose by showing that it is not oxidized by bromine water. He sought to obtain confirmation of this conclusion by reducing turanose with sodium amalgam, since a D-fructosyl-D-glucose, by reduction without side reactions or epimerization, should yield solely a D-fructosyl-D-sorbitol, whereas a D-glucosyl-D-fructose should generate a mixture of a D-glucosyl-D-sorbitol and a D-glucosyl-D-mannitol. The reduction with sodium amalgam was slow and incomplete, and although he obtained some crystalline D-mannitol by the acid hydrolysis of the sirupy product of the reduction, he recognized that side reactions had taken place and that the subject required further study. This problem of the reduction of turanose had to wait for its solution, as will be described later, until the catalytic methods of reduction by hydrogen and palladium or Raney nickel came to supersede reduction by sodium amalgam. Tanret established the formula for melezitose dihydrate,  $C_{18}H_{32}O_{16} \cdot 2H_2O$ , which is the form in which the sugar crystallizes from aqueous solution. He reported that this hydrate is not prominently efflorescent, contrary to previous views. In 10% aqueous solution the dihydrate showed  $[\alpha]_D +83.8^\circ$ , which corresponds to  $+89.3^\circ$  on the anhydrous basis. The melezitose which was used in the research was prepared by him from Alhagi manna and it was carefully purified to free it from some accompanying sucrose. Villiers<sup>6</sup> found sucrose in this manna, but Alekhine found none. Whether the plant exudes both of these sugars is naturally a question of biological interest; a study of it requires that authentic samples be obtained in order to avoid the possibility of sucrose having been added or of the presence of manna from plants other than *Alhagi*. Sherwood and the writer<sup>17</sup> isolated in crystalline form melezitose, sucrose and D-mannitol from a commercial sample of turanjabine.

## VI. THE MANNA OF THE DOUGLAS FIR

In 1918 Miss Helene M. Boas, of the New York Botanical Garden, sent to the writer a sample of manna which had been collected by James A. Teit near Spence's Bridge, British Columbia, from Douglas fir trees (*Pseudotsuga taxifolia* Brit., syn. *P. Douglasii* Carr.). The dry, white, crystalline manna (42.5 g.), in which some small stems and needles of the tree (4 g.) were encrusted, was entirely soluble in water and it proved to consist principally of melezitose.<sup>17</sup> The authors stated that "if the manna can be obtained in large quantities, which appears to be the case, it will indeed furnish an excellent source for melezitose." However, there was found within a few months by the same workers an abundant



FIG. 1.—John Davidson's "specimen of *Pseudotsuga Douglasii*, with exudation of sugar from water-pores at tips of leaves."

and more accessible source for the trisaccharide, as will be described later. Much popular interest was aroused at the time concerning this sugary manna from the Douglas fir, and in 1919 Davidson published<sup>18</sup> a description of its local history together with his views concerning its mode of origin; the following statements are summarized from his article and the report which it mentions.

In the annual report of the British Columbia Botanical Office in Vancouver for 1914, prepared by the Provincial Botanist in charge, John Davidson, there appears an illustration, here reproduced (Fig. 1), showing a branch of Douglas fir laden with white masses of a sugar-like substance; the photograph was prepared from specimens received by the Office from its correspondent James A. Teit, of Spence's Bridge, British Columbia, who, in connection with his ethnological work on the plants

(18) J. Davidson, *The Canadian Field-Naturalist*, 33, 6 (1919).

used as food by the British Columbia Indians, wished to have an explanation of the deposits. The observations of Davidson and Teit seemed to eliminate the possibility that the manna results from the action of insects, or that it is due to any disease or wounding of the trees; it appeared to be a true exudation from the needles under special and very localized conditions of drought, sunlight, soil moisture and temperature, leading to especially high sap-pressure. The quoted subscript for the illustration indicates their idea of the origin of the manna. The manna is well known to the Indians in certain localities but is not regarded by them as of regular annual occurrence. A slight rain is sufficient to dissolve the manna off the trees, and patches of recrystallized sugar may then be found at the base of trees or on the ground. Frequently, however, in this situation it does not recrystallize but may be found in a fluid or semi-fluid condition which is attractive to flies and other insects. Sometimes insects feed on the sugar while it is still on the trees, and it is reported that bears "go after" it, causing the breakage of many branches. The writer adds to the foregoing that the subsequent studies of the entomologist Arnhart on the manna of the larch trees, to be presented in the next section, are suggestive that the origin of the Douglas fir manna may yet be traced to insects; apparently the interesting subject requires further investigation.

## VII. THE OCCURRENCE OF MELEZITOSE IN HONEY

During the time when Sherwood and the writer were analyzing the manna from the Douglas fir, we received a sample of honey from Central Pennsylvania, accompanied by a statement that it had proved unfit winter food for bees and a request that a chemical analysis be made in the hope of disclosing why the bees had died. Bees do not winter well on honeydew honey in general but this material had caused more trouble than usual. The sample had crystallized thoroughly in the comb, and the crystals, representing 10 to 20% of the honey, proved to be pure melezitose.<sup>19</sup> An extensive field investigation of the origin of such honey was made by our colleague Edgar T. Wherry, whose report is included in our article. In summary, the source of this type of honey was the honeydew which bees had collected from a native pine (*Pinus virginiana* Mill.), and the honeydew appeared to be the result of the action of soft-scale insects and aphids on the tissues of the pine. The melezitose of the honey is not made by the bees; the trisaccharide was found in honeydew that was taken from the pine branches. It seems improbable that the soft-scale insects or aphids make the sugar. One is led accordingly

(19) C. S. Hudson and S. F. Sherwood, *J. Am. Chem. Soc.*, **42**, 116 (1920).

to the surmise that melezitose is a constituent of the normal living tissues of plants belonging to such widely different genera as *Pinus*, *Larix*, *Pseudotsuga*, *Alhagi*, *Tilia*, *Populus*, *Laburnum* and *Bambusa*; possibly its presence in the sap of some plant may be detected eventually.

In the years subsequent to 1920, the "melezitose honey" of the Middle Atlantic States has become the principal source of the melezitose that has been used by scientists in further studies of this sugar and turanose. The occurrence of such honey is not regular, as it is associated with local and seasonal periods of drought; the professional apiarists can usually detect it through the observation that the honey crystallizes very soon after the comb cells are capped by the bees, or even occasionally before the capping. The chemist can identify the crystals readily by washing them with glacial acetic acid to remove the sirup, followed by drying and recrystallization; they do not reduce Fehling's solution (distinction from crystals of D-glucose) and their  $[\alpha]_D$  value is about  $+83^\circ$ , as the dihydrate. From the apiary of the United States Department of Agriculture near Washington, D. C., Isbell and Hudson<sup>20</sup> in 1928 obtained 250 kg. of "melezitose honey," which yielded 13 kg. of crystalline melezitose. In 1944 the writer, assisted by Harry W. Diehl, prepared 60 kg. of crystalline melezitose from 600 kg. of "melezitose honey" that was deposited in the apiary of Harold E. Booth, near Charlottesville, Virginia, during a severe drought in the summer of 1943. Another large flow of such honey occurred in the same locality in 1944.

The occurrence of crystalline melezitose in a European honey was established conclusively in 1929 by Nottbohm and Lucius<sup>21</sup>; the source of this honey was the honeydew of linden trees; one recalls that Maquenne isolated melezitose from honeydew of the linden in 1893. In their second article they report crystalline melezitose in a honey (Lärchenhonig), the source of which was honeydew from larch trees of Austria, as reported to them by the entomologist Ludwig Arnhart of Vienna. Two articles by Arnhart<sup>22</sup> are of special importance regarding the cause of the occurrence of larch manna or honeydew. His observations convinced him that the saccharine material is to be attributed to the activity of plant lice of several kinds and he remarks that one can be deceived easily because some species are detectable on larch twigs only by a very careful examination. In some seasons of drought the dry, white manna

(20) H. S. Isbell and C. S. Hudson, see p. 473 of the book "Polarimetry, Saccharimetry and the Sugars," by F. J. Bates and Associates, National Bureau of Standards Circular C440, Washington (1942).

(21) F. E. Nottbohm and F. Lucius, *Z. Untersuch. Lebensm.*, **57**, 549 (1929); **61**, 195 (1931).

(22) L. Arnhart, *Z. angew. Entomol.*, **12**, 457 (1926); **16**, 392 (1930).

becomes very abundant; his description, accompanied by a photograph of a branch with much white manna on it, reminds one of Davidson and Teit's remarks concerning the abundance of the Douglas fir manna on some of those trees and of Bonastre's record that Briançon manna on larch twigs sometimes looks like snow. In view of Arnhart's conclusion, it seems possible that the Douglas fir manna may yet be found to be caused by insects. The origin of the larch manna of Briançon also requires precise study, and likewise that of the Alhagi manna.

### VIII. THE CRYSTALLOGRAPHY OF MELEZITOSE

The following description is taken from the comprehensive measurements of Wherry<sup>23</sup> on melezitose dihydrate.

"The older data as to the crystallography of the trisaccharide melezitose are not only incomplete but contradictory. Villiers<sup>6</sup> described it as monoclinic, bounded by a prism,  $m$ , the base  $p$ , and the side pinacoid  $g_1$ ; he obtained the angles  $g_1:m = 136^\circ 38'$  and  $m:p = 92^\circ 40'$ . On the other hand Alekhine<sup>8</sup> found it to be rhombic, his crystals showing front and side pinacoids,  $o$  and  $a$ , prism,  $m$ , and front and side domes,  $od$  and  $ga$ , although he recorded no measurements at all. In both cases the dihydrate is supposed to have been represented, but no information has been available to indicate whether this was a case of dimorphism or whether the two authors were in fact not dealing with the same substance.

"A sample of melezitose was dissolved in an excess of water and filtered into a small beaker, which was covered with filter paper and allowed to stand at ordinary temperature for several days. The crystals which separated attained a diameter of a millimeter but were crowded together to such an extent as to make them unsuitable for crystallographic measurements. A few minute simple ones were picked out and the balance redissolved by addition of a little water and warming. The liquid was then allowed to cool and the crystals previously picked out were introduced, the vessel being allowed to stand in a room kept at a constant temperature of  $20^\circ$  for several days. The same procedure was followed, using 50% alcohol as the solvent.

"The crystals which separated proved to be rhombic, those from water being broader than those from alcohol; and both types showed the same forms as those described by Alekhine. In the following description his orientation is retained, although the lettering of the forms is changed so as to bring it into accord with modern practice. The faces are somewhat rounded as well as distorted by subparallel intergrowth, so that the measurements vary as much as  $\pm 30'$ , but the general features

of the crystallography can be readily established. The average angular values and the derived axial ratio are presented in Table I.

TABLE I

*Angle-table for Melezitose Dihydrate,  $C_{18}H_{32}O_{16} \cdot 2H_2O$*

System, rhombic, seemingly holohedral, but no doubt bisphenoidal

Axes:  $a:b:c = 1.216:1:0.496$

(Data of Wherry<sup>22</sup>)

Form	Symbol			Description	Observed				Calculated			
	Gdt.	Mill.			$\phi$	$\rho$	$\phi$	$\rho$	$\phi$	$\rho$	$\phi$	$\rho$
<i>b</i>	0	$\infty$	010	Narrow, somewhat dull	0	00	90	00	0	00	90	00
<i>a</i>	$\infty$	0	100	Prominent, brilliant	90	00	90	00	90	00	90	00
<i>m</i>	0	0	110	Narrow, but good	38	05	90	00	—	—	90	00
<i>e</i>	0	1	011	Small, somewhat rounded	0	00	26	30	0	00	26	24
<i>d</i>	1	0	101	Prominent, brilliant	90	00	21	15	90	00	—	—

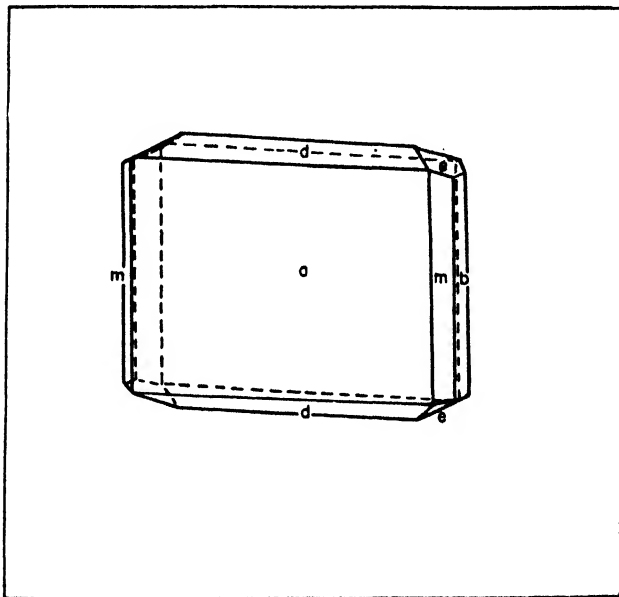


Fig. 2.—Crystal of melezitose dihydrate.

"Many crystals showed only the forms *b*, *a*, and *d*; and it is possible that Villiers' measurements were made on greatly distorted crystals of this development, *b* being his *p*, *a* his *g*<sub>1</sub>, and *d* his *m*. The complete set of forms observed is shown in Fig. 2."

## IX. THE OPTICAL PROPERTIES OF MELEZITOSE

In the same article<sup>23</sup> Wherry describes the optical properties of melezitose as follows:

"Under the microscope melezitose presents the form of plates and rods, in part rectangular in outline and in part terminated by faces inclined at large angles (see Fig. 3). On crushing the crystals, irregular flakes are produced.

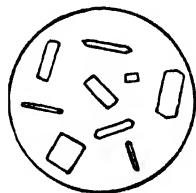


FIG. 3.—Melezitose under the microscope.

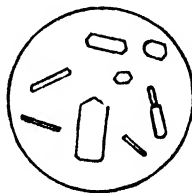


FIG. 4.—D-Glucose under the microscope.

"The rectangular plates yield on immersion in oily liquids of known refractive index the values of  $\beta$  and  $\gamma$ , but crushed fragments usually show values intermediate between  $\alpha$  and  $\beta$  in one direction. Observations were made in light of variable wave length, obtained by a monochromatic illuminator, at 20°. The dispersion relations were found to be as stated in Table II.

TABLE II

*Refractive Indices of Melezitose for Different Wave Lengths*

(Data of Wherry<sup>23</sup>)

Index	Wave Length					
	450	500	550	D, 589	600	650
$\alpha$ .....	1.550	1.546	1.542	1.540	1.540	1.538
$\beta$ .....	1.558	1.553	1.550	1.548	1.547	1.545
$\gamma$ .....	1.561	1.556	1.552	1.550	1.549	1.547
$\gamma - \alpha$ .....	0.011	0.010	0.010	0.010	0.009	0.009

"The refractive indices for sodium light are thus  $\alpha = 1.540$ ,  $\beta = 1.548$ , and  $\gamma = 1.550$ , all  $\pm 0.001$ . The total double refraction is 0.010 but that usually seen is the difference between  $\gamma$  and  $\beta$ , or 0.002.

"In parallel polarized light the double refraction of the plates is seen to be weak, and the colors are mostly brilliant grays of the first order. Rod-like fragments may show white or yellowish colors, corresponding to the maximum double refraction. The extinction is straight and the sign of elongation usually positive.

"In convergent polarized light, on thick plates, a good biaxial interference figure is obtained, the axial angle  $2E_D$  being large,  $85^\circ$ , and the character, negative. The orientation is  $X = a$ ,  $Y = b$ ,  $Z = c$ .

"Certain of these properties, especially the habit and the weak double refraction, may be turned to account in the identification of this sugar in honeys or honeydews. The D-glucose which frequently crystallizes out in honeys is in rods terminated at one end by planes lying  $60^\circ$  apart and rounded at the other end (Fig. 4); and in parallel polarized light showing at least in the centers of the grains brilliant colors of the second order, with negative elongation. If the sirupy honey is removed from around these crystals by glacial acetic acid and the refractive indices are determined by the immersion method, the  $\alpha$  of D-glucose is found to be about one unit in the second decimal place lower than that of melezitose, and the  $\gamma$  a like amount higher. Sucrose can also be readily distinguished from melezitose by its much greater double refraction,  $\alpha$  being 1.45 and  $\gamma$  1.57, and by breaking into irregular fragments with oblique extinction."

#### X. CRYSTALLINE TURANOSE

The crystallization of turanose was first reported by Pacsu and the writer<sup>24</sup> as follows: "In 1918 one of us (H.) found an abundant supply of the rare melezitose in a certain kind of honeydew honey and from it he prepared a small quantity of sirupy turanose in the hope of crystallizing it. Other samples of turanose sirup were prepared subsequently from this stock of melezitose by other workers in the same laboratory. Recently it was observed by D. H. Brauns that one of these sirups, the exact history of which is not now known, had crystallized after standing many years. By the use of these crystals to nucleate turanose sirups which we have lately prepared from melezitose, it has been possible to obtain a rapid crystallization of the sugar in abundant quantities." To this quotation the writer can now add the information, kindly supplied recently by Mr. C. F. Walton, Jr., that Mr. Walton prepared the "other samples of turanose sirup."

Turanose is readily purified by recrystallization from hot methyl alcohol, in which it is moderately, though slowly, soluble. In preparing the sugar, the first stage of the hydrolysis of melezitose by an acid solution is carried out under well controlled conditions, and is followed by the removal of the D-glucose from its mixture with turanose. This is usually accomplished by a selective fermentation with yeast, but an

(24) C. S. Hudson and E. Pacsu, *Science*, **69**, 278 (1929); *J. Am. Chem. Soc.*, **52**, 2519 (1930).



alternative method, in which the D-glucose is oxidized to D-gluconic acid by bromine water and the acid removed as its calcium salt by the use of alcohol, was described. Strains of commercial yeasts vary in their behavior toward turanose; it is our experience that commercial baker's yeast can be used with satisfactory results if the fermentation is stopped after the first vigorous action is over, which requires about twenty-four hours. Even though a sample of yeast may ferment turanose, its action on D-glucose is usually so much more rapid that a selective removal which is sufficiently close for preparative purposes can be attained. Kuhn and Von Grundherr<sup>25</sup> reported that "Löwenbrauerei" yeast fermented D-glucose without any apparent action on turanose.

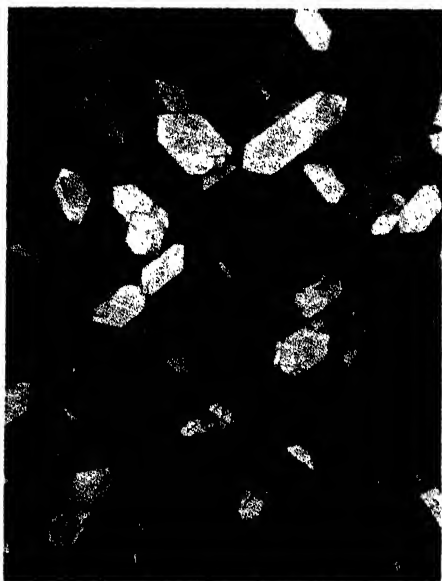


FIG. 5.—Crystals of turanose in polarized light.

(Photographed by Mr. F. P. Phelps of the National Bureau of Standards.)

Turanose crystallizes in well-formed prisms (see Fig. 5); it is anhydrous, shows the composition  $C_{12}H_{22}O_{11}$  by combustion analysis, its melting point is  $157^{\circ}$ , and it exhibits a large and rapid mutarotation in water from an initial  $[\alpha]_D^{22}$  of about  $+22^{\circ}$  to the final value  $+75.3^{\circ}$  in the course of about thirty minutes. Isbell and Pigman<sup>26</sup> found for the mutarotation of turanose at  $20.7^{\circ}$ ,  $[\alpha]_D + 27.3^{\circ}$  (initial)  $\rightarrow +75.8^{\circ}$

(25) R. Kuhn and G. E. Von Grundherr, *Ber.*, 59, 1655 (1926).

(26) H. S. Isbell and W. W. Pigman, *J. Research Natl. Bur. Standards*, 20, 773 (1938).

(final), and at  $0.2^\circ$  the values  $+27.8^\circ$  (initial)  $\rightarrow +70.0^\circ$  (final). The pure crystalline sugar is very sweet. The crystallization of turanose and the abundant supply of melezitose which was on hand from the honeydew honey of the 1928 season, mentioned previously, gave the opportunity for an extensive chemical study of this unusual disaccharide. The investigation was conducted by Professor Pacsu and his students at Princeton University in the years 1931-1939; from these researches and a sequel to them which will be described, has come a precise proof of the structure of turanose.

## XI. THE FIVE TURANOSE OCTAACETATES AND RELATED DERIVATIVES

In introduction to a description of the main results of Pacsu's researches on turanose, it seems advisable for the sake of clarity to describe the views on the structures of melezitose and turanose that were generally accepted when he began his studies, even though parts of those views are now known to have been in error. Kuhn and Von Grundherr<sup>25</sup> had confirmed Georges Tanret's discovery that turanose, which is a reducing sugar, is a ketose and therefore a D-glucosyl-D-fructose rather than a D-fructosyl-D-glucose. Its formula could be written in a generalized way<sup>27</sup> that had come into use, as *glucose* < *fructose* <. Since melezitose is a non-reducing sugar, its formula would be written *glucose* < *fructose* < > *glucose*, if one assumed that all three of its component monoses were of the hemiacetal ring structure, which was highly probable. A cyclic structure from the three components could be excluded, as was inferred by Kuhn and Von Grundherr, because it would contain only nine hydroxyl groups whereas melezitose had been found by Alekhine<sup>8</sup> to yield a hendecaacetate. One sees that the generalized formula for melezitose indicated the possibility that the trisaccharide might be a D-glucosylsucrose because its *fructose* < > *glucose* part might be sucrose. Kuhn and Von Grundherr discussed this possibility and advanced evidence of various kinds, from analogies of hydrolyses of sucrose and melezitose by acids and by enzymes, which seemed at that time to support this view strongly; however, that evidence is recognized today as only suggestive, and in no wise a proof that sucrose is a constituent of melezitose. At this stage of the inquiry, studies on the methylation of melezitose gave new information.

(27) C. S. Hudson, *J. Am. Chem. Soc.*, **38**, 1566 (1916). The symbol < indicates the carbonyl group; its free position on the fructose unit corresponds with the possession of reducing power by turanose. Sucrose is *glucose* < > *fructose*, indicating that the carbonyl groups are united to produce a non-reducing compound sugar.

The full methylation of melezitose in 1926-1927 by Zemplén and Braun<sup>28</sup> and by Miss Leitch<sup>29</sup> yielded a hendecamethyl-melezitose, the acid hydrolysis of which produced two moles of 2,3,4,6-tetramethyl-D-glucopyranose and one mole of a trimethyl-D-fructose, to which a 1,3,4-trimethyl structure was assigned on evidence that cannot now be regarded as precise, though it seemed reasonable at the time. The selection of a faulty formula for this trimethyl-D-fructose, which must have been 1,4,6-trimethyl-D-fructose as will be shown later, led to much confusion during the following decade.

The methylation studies were interpreted by Miss Leitch as indicating that turanose is 6-D-glucopyranosyl-D-fructose (A), but Pacsu<sup>30</sup> called attention four years later to the fact that a 1,3,4-trimethyl-D-fructose could also result from the hydrolysis of methylated melezitose if turanose were a 5-D-glucopyranosyl-D-fructose (B), a formula which Zemplén and Braun had inferred but had changed later to the formula A. Pacsu sought to obtain evidence that might lead to a decision between these two possible conclusions by allowing turanose to react with an excess of triphenylchloromethane; formula A is compatible with the presence of no more than two primary hydroxyl groups in turanose, but formula B could conceivably be compatible with the presence of three of them if turanose should react as a keto form, for example, or as a hemiacetal form with a ring that was not of the pyranose or furanose type. The derivative which he obtained was a tri-(triphenylmethyl)-turanose, which excluded formula A if it be assumed that the triphenylchloromethane had reacted only with primary hydroxyl groups. Pacsu therefore adopted from the previous methylation studies the original formula B of Zemplén and Braun, 5-D-glucopyranosyl-D-fructose, for turanose and he followed this formula for several years in naming the new derivatives which he made. As the work progressed, the number of isomeric octaacetates of turanose, five in total, became larger than could be accounted for by the formula unless some of these were of an orthoacetate, rather than normal, structure, or unless the turanose were reacting in ring forms of which there were no known examples. Thus Pacsu and Cramer<sup>31</sup> came to the conclusion that "in this difficult situation a complete re-investigation of the structure of turanose becomes necessary." In support of their doubts concerning parts of the methylation evidence, they mentioned that the crystalline authentic 1,3,4-trimethyl-D-fructose,

(28) G. Zemplén and G. Braun, *Ber.*, **59**, 2230, 2539 (1926).

(29) Grace C. Leitch, *J. Chem. Soc.*, 588 (1927).

(30) E. Pacsu, *J. Am. Chem. Soc.*, **53**, 3099 (1931).

(31) E. Pacsu and F. B. Cramer, *J. Am. Chem. Soc.*, **59**, 1059 (1937).

which had then come to be known,<sup>32</sup> differed even in the sign of its large rotation from the sirupy products to which Zemplén, Braun and Miss Leitch had ascribed this formula. Pacsu came to the conclusion that turanose was reacting in the furanose, the pyranose, and the keto form, a view which would account readily for the existence of the five octa-

TABLE III  
*Derivatives of Turanose from the Researches of Pacsu and Coworkers*

Substance	Melting Point, °C.	$[\alpha]_D^{20}$	Solvent
$\alpha$ -Turanopyranose octaacetate	194-195	+103.2°	chloroform
$\beta$ -Turanopyranose octaacetate	216-217	+ 20.5	chloroform
$\alpha$ -Turanofuranose octaacetate	158	+107.0	chloroform
$\beta$ -Turanofuranose octaacetate	sirup	+ 67.4	chloroform
<i>keto</i> -Turanose octaacetate	96	+126.2	chloroform
Heptaacetyl- $\beta$ -turanopyranosyl chloride	165	- 0.4	chloroform
Heptaacetyl- $\beta$ -turanopyranosyl bromide	133-134	- 30.5	chloroform
Heptaacetyl- $\beta$ -turanopyranosyl iodide	105-106	- 54.2	chloroform
$\beta$ -Turanopyranose heptaacetate	140-141	+ 37.0	chloroform
Methyl $\beta$ -turanopyranoside heptaacetate	188-189	+ 27.5	chloroform
Methyl $\beta$ -turanopyranoside	173-174	+ 3.6	water
Hexaacetyl- $\beta$ (?)-turanopyranose methyl 1,2-orthoacetate	162-164	+ 80.0	chloroform
$\beta$ (?)-Turanopyranose methyl 1,2-orthoacetate	137	+114.6	water
3- $\alpha$ -D-Glucopyranosyl-D-sorbitol nonaacetate	116.5	+ 70.9	chloroform
3- $\alpha$ -D-Glucopyranosyl-D-mannitol nonaacetate	142	+ 89.3	chloroform

acetates; on such a view, however, the D-glucose unit could not be united with the D-fructose unit at either position 5 or 6 of the latter. Position 4 had come to be eliminated after the structures of maltose and cellobiose were known, since the phenylosazone of turanose is entirely different from those of these sugars. Continuing the investigation, Pacsu, Wilson and Graf<sup>33</sup> synthesized 1- $\beta$ -D-glucopyranosyl-D-fructose and found that it did not give a phenylosazone but appeared to hydrolyze slowly to yield some D-glucose phenylosazone; they concluded that the easy formation of a phenylosazone from turanose indicated that the disaccharide union cannot be at position 1; thus by elimination there remained only position

(32) H. Hibbert, R. S. Tipson and F. Brauns, *Can. J. Research*, **4**, 221 (1931); S. W. Challinor, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 676 (1934).

(33) E. Pacsu, E. J. Wilson, Jr. and L. Graf, *J. Am. Chem. Soc.*, **61**, 2675 (1939); E. Pacsu, *ibid.*, **62**, 2568 (1940).

3. The evidence from studies of the action of enzymes on melezitose and turanose by various workers, to be described later, had led to the general acceptance of the view that turanose, like maltose, is an  $\alpha$ -D-glucoside; accordingly, Pacsu and coworkers were led by the results of their series of researches on turanose<sup>34</sup> to conclude in 1939 that the sugar is  $\beta$ - $\alpha$ -D-glucopyranosyl-D-fructose. A list of turanose derivatives from the researches of Pacsu and his associates is recorded in Table III, which is taken from the article by Pacsu, Wilson and Graf,<sup>33</sup> with some condensations and additions. The structures of several of these derivatives are discussed in Professor Pacsu's article on "Orthoesters of the Sugars" in Volume 1 (1945) of the "*Advances in Carbohydrate Chemistry*."

## XII. STRUCTURAL INTERPRETATION OF THE MUTAROTATION OF TURANOSE

It has been recognized for many years that the deviations from the unimolecular order which are shown by the mutarotations of a few of the aldoses (for example, galactose and arabinose) appear to indicate that a shift between the pyranose and furanose structures accompanies their normal mutarotations. Isbell and Pigman,<sup>35</sup> who have studied this subject extensively with many mutarotating aldoses and ketoses, suggested in 1938 that the mutarotations of fructose and turanose consist of, or involve, such a shift, and that on this hypothesis one may assume that neither of the hydroxyl groups on carbon atoms 5 and 6 of the D-fructose unit of turanose can be concerned in the disaccharide linkage. A linkage at carbon atom 4 had already appeared to be excluded by the non-identity of turanose phenylosazone with the osazone of maltose or of cellobiose, as previously mentioned. They considered position 1 excluded because of the existence of turanose phenylosazone, which they assumed to be of normal osazone structure. Accordingly, they concluded that turanose is "very probably  $\beta$ -D-glucopyranosyl-D-fructose," which was the first published statement that the union was probably at position 3. In continuation, Isbell<sup>36</sup> presented experiments three years later from which he inferred that in strongly alkaline solution turanose hydrolyzes, a behavior which he attributed to its unique character of possessing its D-glucose substituent at carbon atom 3 of its D-fructose unit; thus its

(34) In addition to those already cited, there are the following: E. Pacsu, *J. Am. Chem. Soc.*, **54**, 3649 (1932); **55**, 2451 (1933); E. Pacsu and F. V. Rich, *ibid.*, **55**, 3018 (1933); F. B. Cramer and E. Pacsu, *ibid.*, **59**, 711 (1937).

(35) H. S. Isbell and W. W. Pigman, *J. Research Natl. Bur. Standards*, **20**, 787 (1938).

(36) H. S. Isbell, *J. Research Natl. Bur. Standards*, **26**, 35 (1941).

enolization would result in the production of a structure that presumably would be hydrolyzed by alkali.

### XIII. PROOF OF THE STRUCTURE OF TURANOSE

In 1944 it was shown by the writer<sup>37</sup> from considerations of symmetry that in case turanose is *3- $\alpha$ -D-glucopyranosyl-D-fructose*, it should be possible to obtain from it and from maltose an identical derivative, the identity of which would prove the structure of turanose by direct and conclusive evidence, since the structure of maltose is not in doubt. The essence of this method of correlation lies in the long recognized symmetry of the hexitol mannitol; oxidation of its structure at either end leads to one and the same sugar, mannose. The first and sixth positions of mannitol are equivalent, the second is equivalent with the fifth and the third with the fourth; thus 3-methyl-D-mannitol and 4-methyl-D-mannitol are synonymous names for a single substance. Pacsu and Rich<sup>34</sup> had reduced the keto form of turanose octaacetate by hydrogen and palladium, and by customary reactions had finally derived in pure crystalline condition a substance to which Pacsu, Wilson and Graf<sup>33</sup> assigned the structure *3- $\alpha$ -D-glucopyranosyl-D-sorbitol nonaacetate* (m. p. 116.5° and  $[\alpha]_D^{20} + 70.9^\circ$  in chloroform) and the second product that is to be expected, since turanose is a ketose, *3- $\alpha$ -D-glucopyranosyl-D-mannitol nonaacetate* (m. p. 142° and  $[\alpha]_D^{20} + 89.3^\circ$  in chloroform). Haworth, Hirst and Reynolds<sup>38</sup> had changed maltose, through maltal, to the epimeric epimaltose, a crystalline sugar. Since the structure of maltose was known beyond reasonable doubt<sup>39</sup> through the evidence from methylation studies, enzyme actions and rotatory relationships, to be *4- $\alpha$ -D-glucopyranosyl-D-glucose*, epimaltose must be *4- $\alpha$ -D-glucopyranosyl-D-mannose* and its reduction should give *4- $\alpha$ -D-glucopyranosyl-D-mannitol*, the nonaacetate of which should be identical with the crystalline substance of m. p. 142° that Pacsu and Rich had described, if the postulated structure of turanose be correct. Accordingly, the writer prepared<sup>40</sup> crystalline epimaltose, verified the physical constants which Haworth, Hirst and Reynolds<sup>38</sup> had found for it, reduced it with hydrogen and Raney nickel and acetylated the sirupy material. The nonaacetate of epimaltitol crystallized readily, in high yield, and it proved to be

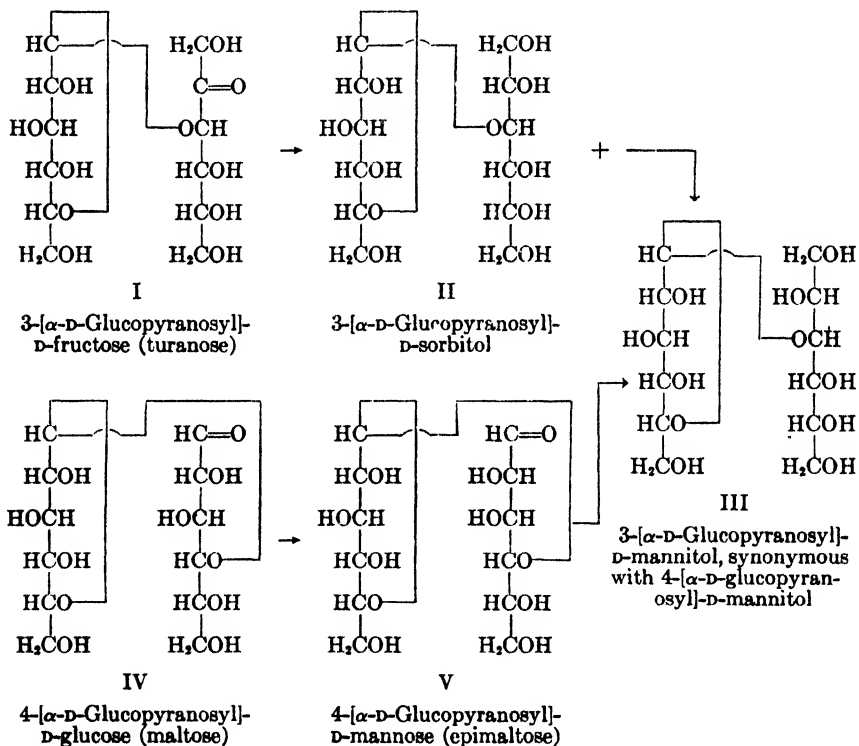
(37) C. S. Hudson, *J. Org. Chem.*, **9**, 117 (1944).

(38) W. N. Haworth, E. L. Hirst and R. J. W. Reynolds, *J. Chem. Soc.*, 302 (1934).

(39) J. C. Irvine and I. M. A. Black, *J. Chem. Soc.*, 862 (1926); C. J. A. Cooper, W. N. Haworth and S. Peat, *ibid.*, 876 (1926); W. N. Haworth and S. Peat, *ibid.*, 3094 (1926); W. N. Haworth, J. V. Loach and C. W. Long, *ibid.*, 3146 (1927).

(40) C. S. Hudson, *J. Org. Chem.*, **9**, 470 (1944).

identical with Pacsu and Rich's product in the turanose series. The rotation of the product from either series was  $+90.8^\circ$  ( $\text{CHCl}_3$ ), in substantial agreement with their value  $+89.3^\circ$ , and the melting point in all cases was  $142^\circ$ . The structure of turanose was thus established conclusively; it is *3- $\alpha$ -D-glucopyranosyl-D-fructose*, as had been inferred by Pacsu and by Isbell and Pigman, from their mutually differing lines of experimental evidence. Its configurational formula is I, expressed in present conventions. The accompanying structural formulas I to V illustrate how it proves possible, because of the equivalence of positions 3 and 4 in mannitol, to obtain from turanose and maltose (through epimaltose) one and the same reduction product.



#### XIV. GENERALIZATION OF THE RELATIONSHIP BETWEEN THE STRUCTURES OF TURANOSE AND MALTOSE

"In" the foregoing proof of the structure of turanose, the generally accepted structure of maltose which has come from the evidence supplied

(41) Quotation from the article of reference 40.

by methylation studies, enzyme actions and rotatory relationships, has been predicated. It seems worthy of notice that the new data showing the identity of the D-glucosyl-D-mannitol from maltose and turanose lead to a new and independent proof for the 4-position of union in maltose and the 3-position in turanose. Referring to the schematic formulas VI to XII, this identity of VI and VII proves rigorously that the D-glucosyl substituent is the same in the two disaccharides ( $R_M = R_T$ ), and in consequence the non-identity of the derived D-glucosyl-D-sorbitols (XI<sup>42</sup> and XII) proves that the position of union is not at the same numbered carbon atom in maltose and turanose and must therefore be at respective positions which become equivalent in mannitol. In the general case that is represented by the formulas, there are three such pairs of equivalent positions in VI and VII, namely, 1-6, 2-5, and 3-4. Introducing at this stage the fact that both maltose and turanose form phenylosazones, it is evident that in maltose the union cannot be at its carbon atoms 1 or 2 and therefore not at 6 or 5 in turanose, and that in turanose it cannot be at its carbon atoms 1 or 2 and therefore not at 6 or 5 in maltose. Only the third pair of positions (carbon atoms 3 and 4) is not excluded; the union in maltose must be at one of these positions and the union in turanose at the other. To distinguish further, additional evidence must be introduced. The degradation of maltose has been shown<sup>43</sup> to lead to a D-glucosyl-D-arabinose that forms a crystalline phenylosazone, the existence of which proves that the union in maltose cannot be at its carbon atom 3 (which becomes the carbon atom 2 of D-glucosyl-D-arabinose); the union in maltose is thus limited to its position 4, and in consequence the union in turanose is restricted to its position 3. The distinction between positions 3 and 4 for the maltose union also follows from the fact<sup>39</sup> that the hydrolysis of fully methylated maltose yields, as one product, 2,3,6-trimethyl-D-glucose, a result which excludes position 3 for the union."

#### XV. PROOF OF THE NORMAL CHARACTER OF THE PHENYLOSAZONES OF TURANOSE AND MALTOSE

It has been assumed in the preceding section that the phenylosazones of turanose, maltose, and D-glucosyl-D-arabinose have structures like that of D-glucose phenylosazone. This normal osazone structure is now proved<sup>40</sup> by the following data for the turanose and maltose osazones, and there can be little doubt that it also holds for the osazone of D-glucosyl-D-arabinose. Turanose phenylosazone has been converted by

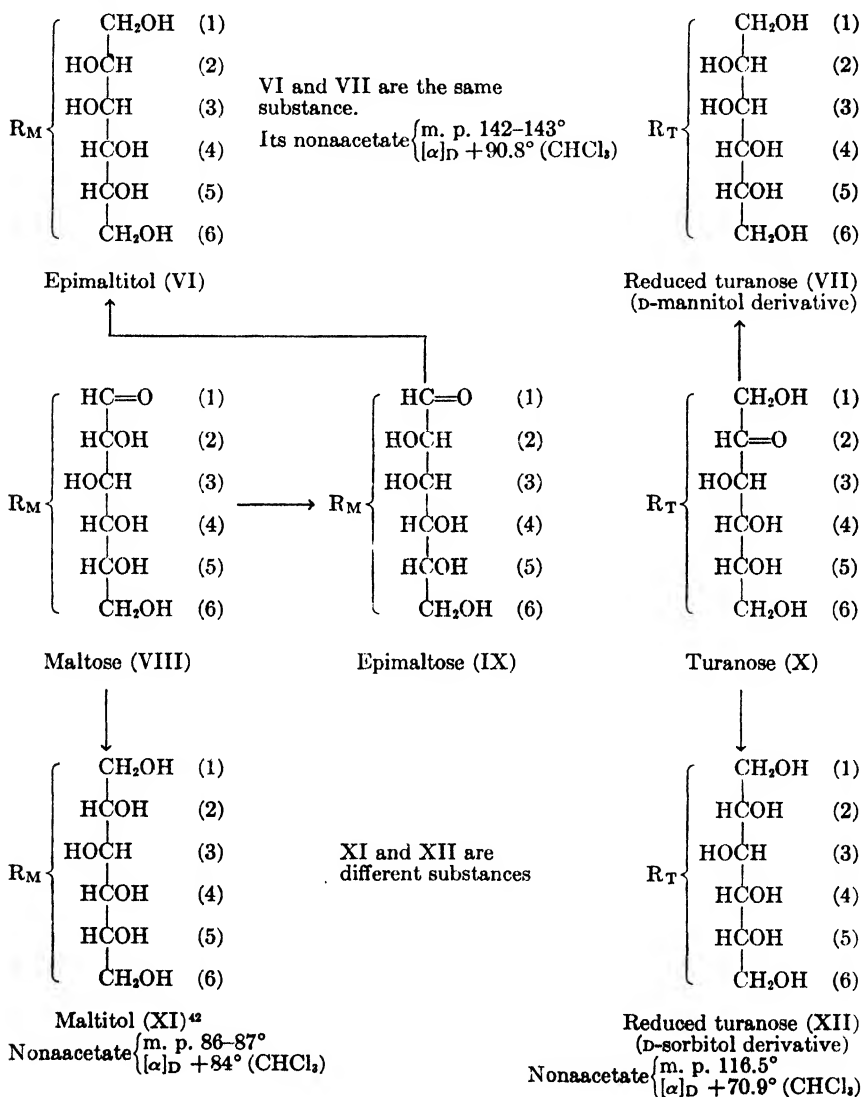
(42) M. L. Wolfrom and T. S. Gardner, *J. Am. Chem. Soc.*, **62**, 2553 (1940).

(43) G. Zemplén, *Ber.*, **60**, 1562 (1927).



*Diagram Illustrating the Generalized Relationship between Turanose and Maltose*

( $R_M$  indicates in a general way the D-glucosyl component of maltose, and  $R_T$  that of turanose. One OH becomes O when a position of union is specified.)

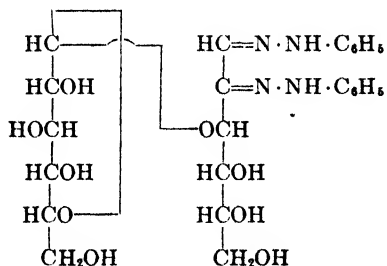


the action of copper sulfate solution to turanose phenylosotriazole, which crystallizes well. The acid hydrolysis of turanose phenylosotriazole yields D-glucose and D-glucose phenylosotriazole, the normal structure of which has been established previously.<sup>44</sup> The phenylosazone of maltose undergoes the usual reaction with copper sulfate solution but the maltose phenylosotriazole has not crystallized; however, the acid hydrolysis of the reaction product produces crystalline D-glucose phenylosotriazole in good yield, which proves the presence of maltose phenylosotriazole in the reaction product and shows the normal character of maltose phenylosazone.

### 1. Further Characterization of Turanose Phenylosazone

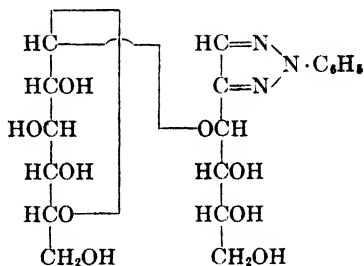
With an abundant supply of pure crystalline turanose available, its phenylosazone (XIII) could be studied in more detail than had been possible in Fischer's investigation of the small quantity of amorphous turanose that he had received from Konowaloff (p. 9).

*Turanose Phenylosazone.* A mixture of 4 g. of turanose, 2 cc. of water, and 1 cc. of phenylhydrazine was warmed on the steam-bath until solution was complete. To the cooled solution was added 3.5 cc. of phenylhydrazine and 4 cc. of glacial acetic acid, and the mixture returned to the steam-bath for one hour. At the expiration of this time, 40 cc. of warm 60% alcohol was added and, upon cooling, a rapid crystallization of the osazone occurred. The osazone was recovered by filtration and washed with absolute alcohol followed by ether to yield 4.2 g. (69%) of lemon-yellow needles. The osazone is soluble in hot water and separates on cooling as jelly-like particles, but water is not a satisfactory solvent for its purification. It was recrystallized from 15 parts of 95% alcohol with good recovery, as needles which melted with decomposition at 200–205° and rotated  $[\alpha]_D^{20} + 24.5^\circ \rightarrow +33.0^\circ$  (24 hours, constant value; c, 0.82) in a mixture of 4 parts of pyridine, by volume, and 6 parts of absolute ethyl alcohol. In methyl cellosolve (ethylene glycol monomethyl ether) solution it rotated  $[\alpha]_D^{20} + 44.3^\circ \rightarrow + 48.5^\circ$  (24 hours, constant value; c, 0.80).



XIII

Turanose phenylosazone



XIV

Turanose phenylosotriazole

## 2. Preparation and Acid Hydrolysis of Turanose Phenylsotriazole

Turanose phenylsotriazole, the configurational formula of which is XIV, was prepared from turanose phenylsazone.

*Turanose Phenylsotriazole.* A solution of 15 g. of turanose phenylsazone in 300 cc. of hot water was placed on the steam-bath and a solution of 22 g. of copper sulfate pentahydrate in 150 cc. of hot water was added. The mixture turned a deep cherry-red at once and in a short time (fifteen min.) a red precipitate had formed and the solution had become green. After thirty minutes from the time of addition of the copper solution, the solution was cooled, filtered, and the copper removed as sulfide. The clear light yellow filtrate was neutralized with 45 g. of barium carbonate and the insoluble material removed by filtration. The filtrate was extracted with five 50-cc. portions of ether to remove the aniline, and the aqueous portion was concentrated *in vacuo* to a thick sirup. The sirup was dissolved in 60 cc. of warm alcohol, filtered to remove a slight turbidity and diluted with 65 cc. of ether. Upon cooling and scratching, the product crystallized as large prisms; yield 8.9 g. (72%). The phenylsotriazole was recrystallized from 10 parts of alcohol and when pure showed the melting point 193–194° and rotated  $[\alpha]_D^{20} + 74.5^\circ$  in aqueous solution (c, 0.90).

*Acid Hydrolysis of Turanose Phenylsotriazole.* A solution of 2 g. of turanose phenylsotriazole in 100 cc. of 0.5 *N* hydrochloric acid was heated in a boiling water-bath at 99° for six hours. After cooling to 5° for several hours, the needle crystals of *D*-glucose phenylsotriazole were removed by filtration and washed with cold water; yield 1.17 g. (94%); m. p. 195–196°, rotation  $[\alpha]_D^{20} - 81.3^\circ$  in pyridine solution (c, 0.83), in agreement with the values for pure *D*-glucose phenylsotriazole.<sup>4</sup> The aqueous filtrate from the *D*-glucose phenylsotriazole precipitate was neutralized with 10 g. of silver carbonate, filtered, the excess silver ion removed as the sulfide, and the clear filtrate from the sulfide precipitate concentrated *in vacuo* to a thick sirup. The sirup was taken up in 5 cc. of warm methanol, filtered to remove a slight turbidity and re-concentrated to a thin sirup which, upon addition of an equal volume of glacial acetic acid, crystallized; yield 0.50 g. (60%). The crystals were shown to be *D*-glucose by their equilibrium rotation  $[\alpha]_D^{20}$  in aqueous solution (+ 52.5°).

## XVI. DISCUSSION OF THE STRUCTURE OF MELEZITOSE

### 1. The Inconclusive Character of the Past Evidence relating Melezitose with Sucrose

The establishment of the structure of turanose furnishes a firm basis for an eventual determination of the structure of melezitose. One can start with the assurance that the trisaccharide is 3-[ $\alpha$ -*D*-glucopyranosyl]-*D*-fructose < > *D*-glucopyranose; this formula requires three further specifications. The first of these is an allocation of a ring to the fructose unit, the second is a decision between the  $\alpha$ - and  $\beta$ -forms for this unit, and the third is a similar decision between these forms for the right-hand *D*-glucopyranose unit. Decision on these three matters can be made readily if sucrose should ever be found to result from a partial hydrolysis of melezitose, because sucrose is almost certainly  $\beta$ -*D*-fructo-

*furanose* < >  $\alpha$ -D-glucopyranose<sup>45</sup>; melezitose would then be 3-[ $\alpha$ -D-glucopyranosyl]- $\beta$ -D-fructofuranosyl- $\alpha$ -D-glucopyranoside. This attractive proof has never been realized; no non-reducing disaccharide has been found to result from a hydrolysis of melezitose either by acids or enzymes. Acid hydrolysis breaks the right-hand union far faster than it does the left, a behavior which led Alekhine, who first observed it, to his discovery of turanose. Ever since the time of Georges Tanret's discovery that turanose is a D-glucosyl-D-fructose, chemists have recognized the possibility that melezitose may be a D-glucose < sucrose. Raffinose has been proved to be of this type (namely, D-galactose < sucrose) because almond emulsin, which contains the enzyme  $\alpha$ -D-galactosidase, hydrolyzes it to D-galactose and sucrose<sup>46</sup>; gentianose has been shown to be a D-glucose < sucrose because it has been hydrolyzed by almond emulsin, which contains also the enzyme  $\beta$ -D-glucosidase, to D-glucose and sucrose.<sup>47</sup> The sucrose was isolated in both cases in pure crystalline form. The surmise that melezitose is also a substituted sucrose is thus a very natural one, and has a good degree of probability from the biological viewpoint, because no D-fructose < > D-glucose disaccharide has ever been found in nature that is not  $\beta$ -D-fructofuranose < >  $\alpha$ -D-glucopyranose (namely, sucrose). In the absence of direct proof, chemists regarded over a long period of years the ease of hydrolysis of one of the unions in melezitose as almost conclusive evidence that a "sucrose union" is present; but this behavior lost all value as evidence when it was discovered that methyl D-fructopyranoside is hydrolyzed by acids at about the same fast speed as is methyl D-fructofuranoside.<sup>46</sup> Moreover, this ease of hydrolysis never was evidence concerning the  $\alpha$ - or  $\beta$ -configuration of the D-fructose unit in melezitose. The fact that purified preparations of invertase, which hydrolyze sucrose, raffinose and gentianose rapidly, are without any action on melezitose seemed for many years difficult to reconcile with the view that melezitose contains a "sucrose union." Although Richard Kuhn was able later to account for this on a reasonable hypothesis, which will be discussed later and shown to have been very fruitful, his work did not prove the existence of a "sucrose union," but only removed an important objection to it. The general acceptance of the very natural hypothesis that melezitose is a D-glucosyl-sucrose has led to positive statements in many scholarly books and research articles implying that this structure is fully established. Such is not the case, as will be clear from the continuation. The full structure of melezitose

(45) C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.*, **59**, 1170 (1937).

(46) C. Neuberg, *Biochem. Z.*, **3**, 519 (1907).

(47) E. Bourquelot and M. Bridel, *Compt. rend.*, **171**, 11 (1920).

remains to be established by experimental evidence. The evidence that is available at the present time will now be presented.

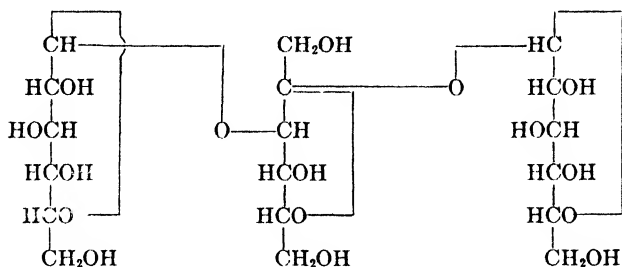
## 2. Revised Interpretation of the Methylation Data concerning Melezitose

Consider first the question of the ring structure of the D-fructose unit of melezitose. Is its ring of the pyranose type or is it furanose? Miss Leitch<sup>29</sup> showed that the trimethyl-D-fructose which was one of the scission products from fully methylated melezitose, yielded by its subsequent methylation a methyl tetramethyl-D-fructoside which gave the previously known 1,3,4,6-tetramethyl-D-fructofuranose by acid hydrolysis. The identification which she made for the tetramethyl-D-fructose remains valid today, and her experimental data establish the relationship of this substance to the particular trimethyl-D-fructose that resulted from the hydrolysis of hendecamethyl-melezitose. Obviously this trimethyl-D-fructose carried no methyl group at position 5. These conclusions by Miss Leitch have not been affected by later developments, though these developments have changed other aspects of the early research. She postulated the 1,3,4 structure for this trimethyl-D-fructose and inferred that its observed methylation to the known 1,3,4,6-tetramethyl-D-fructose established a furanose ring for the D-fructose unit of melezitose. As was shown later by Pacsu (page 20), the inferred 1,3,4 structure was also compatible with the selection of a pyranose ring type for this unit of melezitose, it being understood of course that Pacsu's argument involved the view then generally held that the union in turanose was at position 5 or 6 of the D-fructose. At the present time the situation with regard to this argument has changed completely, now that the union in turanose is known to be at position 3. Obviously, the trimethyl-D-fructose cannot have been the 1,3,4 form, but, since it carried no methyl groups at positions 5 (already mentioned) and 3, it must have been 1,4,6-trimethyl-D-fructose. This revised interpretation of Miss Leitch's data results in the valid assignment of a furanose ring to the D-fructose portion of melezitose, which can now be designated 3-( $\alpha$ -D-glucopyranosyl)-D-fructofuranose < > D-glucopyranose.

## 3. The Oxidation of Melezitose by Per-iodic Acid

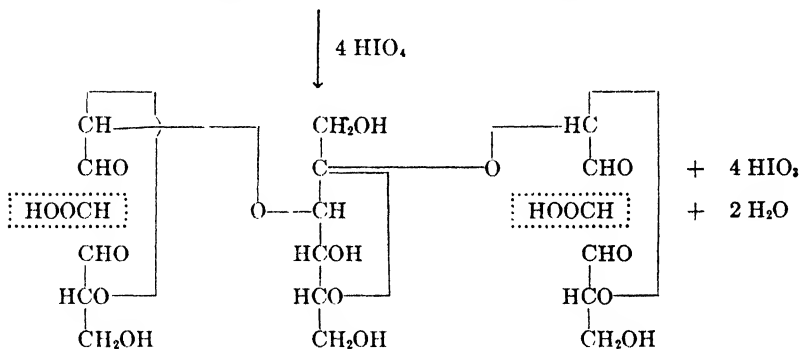
The presence of the pyranose ring in the two D-glucose units of melezitose was inferred by Zemplén and Braun from good evidence, and made certain by Miss Leitch<sup>29</sup> through the crystallization of the requisite amount of 2,3,4,6-tetramethyl-D-glucose from the scission products of the fully methylated trisaccharide. The revised interpretation of Miss Leitch's experimental data concerns only the structure of the

D-fructose unit, which is now established as of the furanose type. A configurational formula for melezitose that is limited to the expression of these ring structures and leaves open the matter of  $\alpha, \beta$  classification is shown by XV.



Melezitose (XV)

(3-(D-Glucopyranosyl)-D-fructofuranose < > D-glucopyranose)



XVI

Tetraaldehyde from melezitose (not isolated)

Recent work by Richtmyer and the writer<sup>48</sup> has confirmed this formula XV through a study of the oxidation of melezitose by per-iodic acid. They found that the oxidation resulted in the reduction of 4 moles of the acid to 4 moles of iodic acid for each mole of sugar, and the generation of 2 moles of formic acid; no formaldehyde could be detected. It can be shown that these analytical measurements lead to this formula by an independent conclusive proof.

The absence of formaldehyde from the oxidation products proves (1) that in the case of the D-fructose unit its ring must be of the furanose or pyranose type; and (2) that in the case of the D-glucose units their rings are

(48) N. K. Richtmyer and C. S. Hudson, *J. Org. Chem.*, 11, 610 (1946).

*restricted to pyranose or septanose forms.* (The septanose ring would have an oxygen atom uniting carbon atoms one and six, making a seven-atom ring, as distinguished from the pyranose (six-atom) and furanose (five-atom) rings.)

With conclusions (1) and (2) in mind, consider the possibility that each D-glucose unit may possess a septanose ring; the oxidation of the hydroxyl groups on the second, third, fourth and fifth carbon atoms of each D-glucose unit would reduce in total 6 moles of per-iodic acid and generate 4 moles of formic acid, values which exceed the experimental data. If one D-glucose unit were of the septanose ring type and the second of the pyranose, 5 moles of per-iodic acid would be reduced by these D-glucose units and 3 moles of formic acid generated; again these values exceed the experimental data. The measurements are compatible only with the assumption of pyranose rings for both D-glucose units; this assumption corresponds with four moles of per-iodic acid consumed and two moles of formic acid produced. The fact that the requirement for these two units corresponds with the experimental measurement indicates that the D-fructose unit is not oxidized by the per-iodic acid and must therefore be of the furanose ring type, containing no pair of hydroxyl groups on contiguous carbon atoms. Experiment<sup>48</sup> shows that the oxidation of melezitose by four moles of per-iodic acid leaves its D-fructose unit essentially unattacked: by customary subsequent oxidation with bromine water of the tetraaldehyde from melezitose (XVI), followed by acid hydrolysis, the presence of free D-fructose in the levorotatory solution has been demonstrated through the preparation of D-glucose phenylosazone in good yield. In view of the analytical measurements that have been discussed, it is evident that this osazone must have originated from D-fructose rather than from D-glucose; in confirmation, D-fructose was also identified in another aliquot through its characteristic *p*-nitrophenylhydrazone.

One notices that the data from the oxidation of melezitose by per-iodic acid confirm the pyranose structure of the D-glucose unit in turanose, and therefore also in the case of maltose, in agreement with the original assignments for both of these disaccharides from methylation studies.

#### 4. *The Action of Enzymes on Melezitose*

The augmented formula of p. 30 for melezitose,  $\beta$ -[ $\alpha$ -D-glucopyranosyl]-D-fructofuranose  $\leftrightarrow$  D-glucopyranose, lacks only the two pertinent  $\alpha, \beta$  allocations. In the case of the D-glucopyranose unit there is evidence from enzyme studies, which seems conclusive, that it is of the  $\alpha$ -D-type, as in sucrose. First, there is the evidence of a negative kind that the

$\beta$ -D-glucosidase of almond emulsin is without action on melezitose<sup>26</sup> or turanose.<sup>25, 49</sup> Next there is the positive evidence that the enzyme  $\alpha$ -D-glucosidase which is obtainable from yeast, malt, *Aspergillus oryzae* and some other organisms, hydrolyzes both turanose<sup>49</sup> and melezitose, the latter to its three components.<sup>50</sup> The action of enzymes thus discloses that both D-glucose units in melezitose are of the  $\alpha$ -D-type, and melezitose becomes  $\beta$ -[ $\alpha$ -D-glucopyranosyl]-D-fructofuranose  $< >$   $\alpha$ -D-glucopyranose.

The details of the evidence from enzyme studies that have finally led to the foregoing summary statements need not be presented here, as they have been set forth in a thorough review by Rudolf Weidenhagen,<sup>51</sup> whose experimental and theoretical studies in this field have reconciled the numerous conflicting observations that beclouded the subject during many years. The first clarifying idea in this complicated study was the inference by Richard Kuhn<sup>52</sup> that there exist two enzymes which can hydrolyze sucrose; they are referred to now as  $\alpha$ -D-glucosidase and  $\beta$ -D-fructofuranosidase. Some yeast extracts contain both enzymes and in consequence they liberate the  $\alpha$ -D-glucopyranose units from the melezitose molecule and thereby cause total hydrolysis. On the other hand, the usual purified invertase solutions from yeast contain no  $\alpha$ -D-glucosidase, which disappears during the processes of purification, and they owe their ability to hydrolyze sucrose to their content of  $\beta$ -D-fructofuranosidase; their long-known inability to attack melezitose was ascribed by Kuhn and Von Grundherr,<sup>25</sup> and by Weidenhagen<sup>50</sup> in the subsequent development of the subject, to the alteration of the D-fructose unit of melezitose by the attachment of a D-glucose molecule to it, forming the turanose unit. These well substantiated views have been accepted generally as proving that both D-glucopyranose units of melezitose are of the  $\alpha$ -D-type; that is as far as this evidence can now go and there is no ground for accepting it as even an indication that the D-fructofuranose unit in melezitose is of the  $\beta$ - rather than the  $\alpha$ -configuration. The reason lies in the fact that the lack of action of  $\beta$ -D-fructofuranosidase on melezitose is of the nature of negative evidence, and from it there cannot be drawn the conclusion that an  $\alpha$ -configuration is excluded for the D-fructofuranose unit. This is especially apparent when one con-

(49) M. Bridel and T. Aagaard, *Compt. rend.*, **184**, 1667 (1927); *Bull. soc. chim. biol.*, **9**, 884 (1927); T. Aagaard, *Tids. Kjem. Bergvesen*, **8**, 5, 16, 35 (1828); *C. A.*, **24**, 1089 (1930).

(50) R. Weidenhagen, *Z. Ver. deut. Zucker-Ind.*, **78**, 781 (1928); **80**, 383 (1930).

(51) F. F. Nord and R. Weidenhagen, "Handbuch der Enzymologie," Akademische Verlagsgesellschaft, Leipzig, pp. 512-572 (1940).

(52) R. Kuhn, *Z. physiol. Chem.*, **129**, 57 (1923).

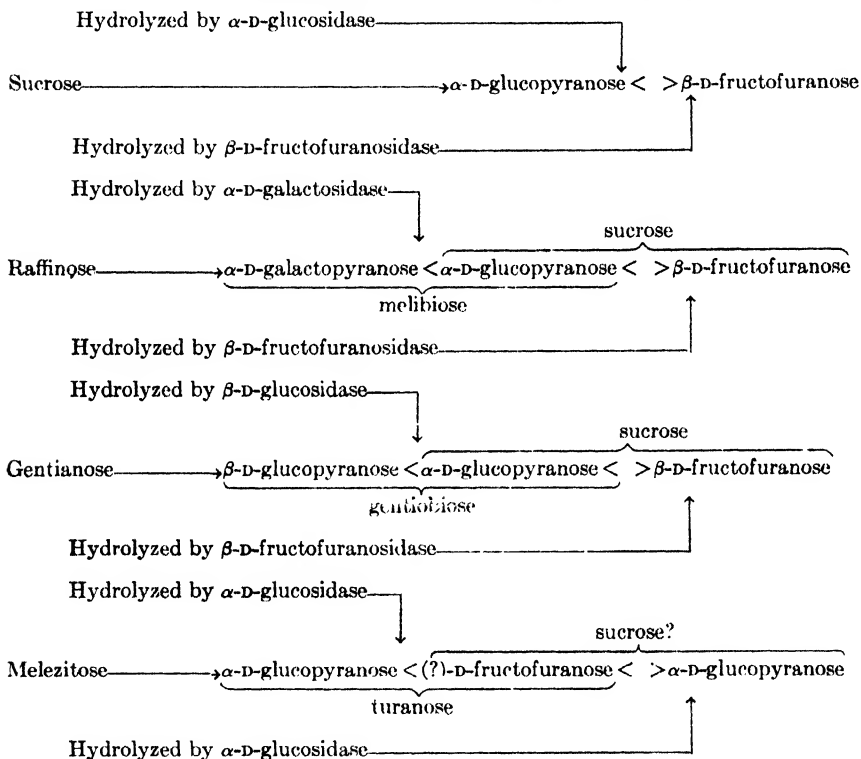


siders the fact that strong solutions of purified invertase, which hydrolyze methyl  $\beta$ -D-fructofuranoside rapidly, have no action on methyl  $\alpha$ -D-fructofuranoside.<sup>53</sup>

The rather complicated enzymic hydrolyses of three of the trisaccharides receive a clear interpretation through the relationships that are shown in Table IV.

TABLE IV

*The Related Enzymic Hydrolyses of Certain Compound Sugars*



The non-reducing disaccharide unit of raffinose and gentianose is certainly sucrose, as previously mentioned; in the case of melezitose all that can be said with assurance at the present time is that this sugar is  $\beta$ -[ $\alpha$ -D-glucopyranosyl]-(?) -D-fructofuranosyl- $\alpha$ -D-glucopyranoside. It would indeed be surprising, from biological views, if the now unknown disaccharide component should eventually prove to be other than sucrose,

(53) C. B. Purves and C. S. Hudson, *J. Am. Chem. Soc.*, **56**, 702, 708, 1969 (1934).

but none of the existing experimental evidence has any bearing upon the decisive last question of the  $\alpha$ - or  $\beta$ -allocation for the D-fructofuranose unit.

### XVII. THE MONOHYDRATE OF MELEZITOSE

Georges Tanret<sup>18</sup> established the composition of the hydrate of melezitose which crystallizes so readily from aqueous solutions as large clear crystals which can become a centimeter on edge if grown slowly; it is the dihydrate,  $C_{18}H_{32}O_{16} \cdot 2H_2O$ . Berthelot<sup>4</sup> and later investigators described its efflorescence as occurring easily, but Tanret believed that the dihydrate is fully stable in air at room temperature. He states that the crystals of melezitose are in general more or less opaque and that this opacity has led to the belief that they effloresce in air. His experience led him to conclude that efflorescence really does not occur at room temperature in air.

Recently Richtmyer and the writer<sup>48</sup> grew some large clear crystals of melezitose dihydrate from an aqueous solution of carefully purified melezitose. When the faces were cleaned of mother liquor by absorption with filter paper, the crystals were not opaque, retained their clear appearance for hours, there was no loss of weight, and the water content could be determined with accuracy, as shown by the following data. After one day, one-half of the sample of clear crystals was powdered and left in the air at room temperature; efflorescence was rapid and within three days the decrease in weight reached a constant value of 3.35% corresponding to the loss of one molecule of water of crystallization from melezitose dihydrate (theory, 3.33%). Meanwhile, on the faces of the other half of the sample of large clear crystals, localized white spots appeared and in the course of several days grew until the whole mass of each crystal became a white solid, the change having progressed throughout the crystal. The loss in weight reached constancy at 3.5%. There was no crumbling, the white crystals preserved the original faces very well and showed a shiny luster. The air-dried powdered material had the composition of melezitose monohydrate,  $C_{18}H_{32}O_{16} \cdot H_2O$ , as was shown by its 3.45% loss in weight (theory, 3.45%) when dried in a vacuum at 110°. Anhydrous melezitose thus obtained is unstable in air and quickly reverts to the monohydrate, the weight of which then remains constant. Melezitose monohydrate is a definite chemical compound of complete stability in the air; its use for the preparation of melezitose solutions of accurate composition can be recommended.

## XVIII. THE SPECIFIC ROTATION OF MELEZITOSE

Most of the values reported for the specific rotation of melezitose lie between  $[\alpha]_D +88.1^\circ$  and  $+88.8^\circ$ , as reckoned for anhydrous melezitose by the observers. Richtmyer and the writer<sup>48</sup> found  $[\alpha]_D^{20} +88.5^\circ$  in water (*c*, 2) for melezitose monohydrate,  $C_{18}H_{32}O_{16} \cdot H_2O$ . Their experience showed that powdered melezitose dihydrate and anhydrous melezitose are transformed rapidly in the air to the stable monohydrate; it thus seems doubtful whether the water content of the samples in the older studies was accurately known, and it now appears likely that the samples were essentially melezitose monohydrate. The value  $[\alpha]_D^{20} +88.5^\circ$  for melezitose monohydrate corresponds to  $[\alpha]_D^{20} +91.7^\circ$  for anhydrous melezitose, and to  $[\alpha]_D^{20} +85.6^\circ$  for melezitose dihydrate.

# THE CHEMISTRY OF ANHYDRO SUGARS

BY STANLEY PEAT

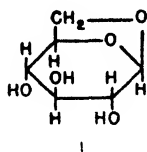
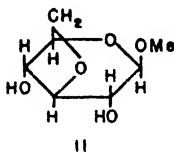
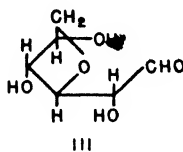
*A. E. Hills Laboratories, The University, Birmingham, England*

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## I. INTRODUCTION

It had long been known that the dry distillation of D-glucose produced in small yield a compound the molecular formula of which was  $C_6H_{10}O_5$ . The name glucose anhydride for this product was abbreviated to glucosan and the fact that it was dextrorotatory recorded.<sup>1</sup> When in 1894, Tanret<sup>2</sup> prepared a glucose anhydride which was levorotatory the name levoglucosan was used to distinguish this compound from the ill-defined glucosan produced by heating glucose to  $170^\circ$ . Levoglucosan is a stable, crystalline compound with a sharp melting point and it sublimes unchanged in a vacuum. The loss of water in its formation from D-glucose involves the disappearance of the reducing properties of the sugar, and in aqueous solution levoglucosan shows no mutarotation. D-Glucose is regenerated from it when it is heated with dilute aqueous acid; it forms a crystalline triacetate and tribenzoate and when the triacetate is treated with bromine and phosphorus the well known 1,6-dibromo-D-glucose triacetate results. From these facts, it is clear that levoglucosan has a dicyclic structure, the anhydrobridge involving the reducing group C1 and the terminal group C6. An atom model indicates that in this dicyclic structure the reducing group of the sugar will have the  $\beta$ -D-configuration. The systematic name for levoglucosan will be therefore 1,6-anhydro- $\beta$ -D-glucopyranose (I).

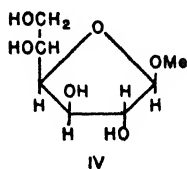
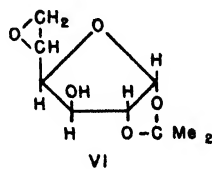
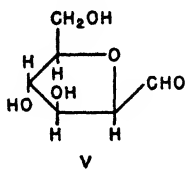
I  
LevoglucosanII  
Methyl 3,6-anhydro- $\beta$ -D-glucopyranosideIII  
3,6-Anhydro-D-glucose

It is to be noticed that although the anhydro ring of levoglucosan is stable to alkali (Tanret's preparative method consisted in the heating of aromatic  $\beta$ -D-glucosides with aqueous alkali), it readily undergoes scission in the presence of acid. An isomeric glucose anhydride prepared in 1912 by E. Fischer showed, however, very different properties.

Fischer, having succeeded in replacing the bromine atom in methyl 6-bromo-triacetyl- $\beta$ -D-glucoside by the amino group, endeavored in collaboration with Zach<sup>3</sup> to effect the removal of the bromine, as well as the acetyl groups, by the treatment of the compound with barium hydroxide,

(1) A. Gélis, *Compt. rend.*, 51, 331 (1860).(2) C. Tanret, *Compt. rend.*, 119, 158 (1894).(3) E. Fischer and K. Zach, *Ber.*, 45, 456 (1912).

in the expectation of regenerating methyl  $\beta$ -D-glucoside ( $C_7H_{14}O_6$ ). Instead, the sole product was a distillable liquid having the composition and the properties of a methyl anhydro-D-glucoside ( $C_7H_{12}O_5$ ). Acid hydrolysis of this glucoside yielded a crystalline anhydro-D-glucose which was isomeric, but not identical, with levoglucosan. The new D-glucose anhydride, unlike levoglucosan, showed reducing properties, formed an anhydro hexose phenylosazone and was characterized by the extreme stability of the anhydro ring toward either acid or alkaline hydrolyzing agents and toward oxidizing or reducing agents. Obviously, the reducing group (C1) of the original glucose is not involved in the anhydro linkage. In point of fact, Fischer's provisional description of the substance as 3,6-anhydro-D-glucose has been amply confirmed by later evidence. Inspection of the formula III indicates that the stable anhydro ring in 3,6-anhydro-D-glucose and in the corresponding methyl  $\beta$ -D-glucoside (II) is actually a hydrated furan ring. It will therefore be described here as a "hydrofuranol" ring. This is not to be confused with the five-membered ring of a normal furanoside as in IV. It is indeed possible for a hydrofuranol ring and a furanoside ring to exist together in the same molecule, as in methyl 3,6-anhydro- $\beta$ -D-glucufuranoside. The properties of the two rings are, however, very different. 3,6-Anhydro-D-glucose was not the first sugar anhydride of the hydrofuranol type to be prepared. Two decades earlier, Fischer and Tiemann<sup>4</sup> had produced, by the deamination of D-glucosamine, a reducing anhydrohexose to which the name chitose was given. Chitose is a liquid with ill-defined properties. It contains a hydrofuranol ring, the oxygen bridge in this case engaging C2 and C5. As will be shown later, there is reason to believe that the deamination is accompanied by optical inversion on the asymmetric atom concerned, in this case C2. Chitose is therefore probably 2,5-anhydro-D-mannose (V).

Methyl  $\beta$ -D-glucufuranoside

3,6-Anhydro-D-glucose resulted, as mentioned, from the hydrolytic removal of a halogen atom at C6. Similar hydrolytic scission of sugar esters of carboxylic acids takes place without dehydration and with the liberation of the normal sugar. Thus, saponification of 6-benzoyl- or

(4) E. Fischer and F. Tiemann, *Ber.*, 27, 138 (1894).

6-acetyl-D-glucose regenerates D-glucose. On the other hand, the hydrolysis of sugar esters of mineral acids such as sulfuric, phosphoric or nitric acids, or of sulfonic acids leads simultaneously to anhydro ring formation if a second suitably placed hydroxyl group is available in the molecule. For example, Ohle and coworkers<sup>5</sup> were able to prepare 3,6-anhydro-D-glucose by the saponification of a derivative of D-glucose 6-*p*-toluenesulfonate (6-tosyl derivative).

During a study of the hydrolysis of sugar *p*-toluenesulfonates, a third type of anhydro ring closure came to light. In this type, the oxygen atom bridges two adjacent carbon atoms and it is therefore designated the ethylene oxide type. The first true example of this type to be prepared was 5,6-anhydro-1,2-isopropylidene-D-glucofuranose (VI) which was prepared by Freudenberg and collaborators<sup>6</sup> in 1928 from 6-bromo-isopropylidene-D-glucose, and by Ohle and Vargha<sup>7</sup> a year later from the 6-tosyl derivative. Earlier examples of sugars containing the three-membered anhydro ring were known, but these were in a special category inasmuch as the oxygen bridge engaged the reducing group (C1), and the anhydro sugars partook of the properties of levoglucosan, and could so be regarded as "glycosans." A representative member of this class is the 1,2-anhydro-D-glucose of Brigl.<sup>8</sup>

## II. CLASSIFICATION

In a classification of the sugar anhydrides it will be convenient to differentiate the compounds in the first place according to the size of the anhydro ring and refer to the five-membered ring or hydrofuranol type and the three-membered ring or ethylene oxide type. It has become customary to class together those anhydrides in which the reducing group (C1) is a member of the anhydro ring under the generic name of glycosan, irrespective of the size of the ring. It will be useful to follow this custom in regard to the naming of this special group but the reviewer prefers to use, in the description of individual members of the series, a uniform system applicable to all classes of anhydrides. Thus Brigl's compound will be named 1,2-anhydro-D-glucose; levoglucosan will be named 1,6-anhydro-D-glucopyranose; etc.

The two main classes of anhydro sugars are therefore the ethylene oxide and the hydrofuranol types. Other anhydro ring systems are known but the number of representatives of such systems is not great.

(5) H. Ohle, L. v. Vargha and H. Erlbach, *Ber.*, 61B, 1211 (1928).

(6) K. Freudenberg, H. Toepfer and C. C. Anderson, *Ber.*, 61B, 1751 (1928).

(7) H. Ohle and L. v. Vargha, *Ber.*, 62B, 2435 (1929).

(8) P. Brigl, *Z. physiol. Chem.*, 121, 245 (1922).

The most common is the seven-membered ring type represented by levo-glucosan. Examples have been recorded of a fourth type, namely that in which the anhydro ring is four-membered, but only two of these examples are well authenticated.<sup>9,10</sup>

In this review, therefore, the following classes of sugar anhydrides will be considered:

*Ethylene Oxide Type-*

2,3-, 3,4-, 4,5-, 5,6-anhydrides.

*Hydrofuranol or Butylene Oxide Type*

2,5- and 3,6-anhydrides.

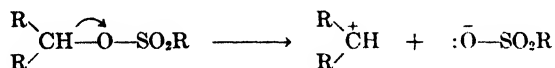
*Glycosan Type*

Three-, four-, five- and seven-membered rings.

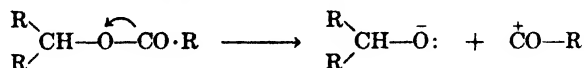
Such sugar anhydrides and their derivatives as have been described in the literature to the year 1944 are included in Tables II, III, IV, V and VI and are classified under the above groups.

### III. THE MECHANISM OF ANHYDRO RING FORMATION

In surveying possible modes of reaction leading to the elimination of the elements of water from a monosaccharide molecule, the attention is at the outset focused on one significant fact. The saponification of the sugar ester of a mineral acid or of a sulfonic acid constitutes one of the chief methods of preparation of anhydro sugars, whereas there is no known example of water elimination accompanying the hydrolysis of the carboxylic acid ester of a sugar (for this purpose, halides are regarded as esters of the halogen acids). It is now generally recognized that the fundamentally different course followed in the hydrolysis of a carboxylic ester and, for example, a sulfonic ester lies in the fact that a preliminary stage in the hydrolysis of the latter results in the appearance of a carbonium cation, thus



A carboxylic ester, however, undergoes scission in such a manner that the oxygen atom remains attached preferentially to the alkyl carbon.

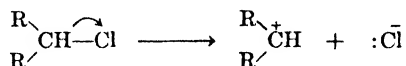


(9) P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, 102, 331 (1933).

(10) R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, 63, 2241 (1941)



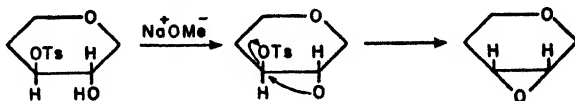
What has been said about sulfonic esters applies also to sulfuric, phosphoric and nitric esters and of course the hydrolysis of halides must necessarily always yield a carbonium cation.



*Definition of Cationogen.* For convenience of description it is obvious that a name is desirable which will include, in a generic sense, all those groups or atoms the removal of which from their combination with carbon denudes the carbon atom of the bonding electron pair and thus generates a carbonium cation. For want of a better word, we would suggest the term "cationogen" to describe such electrophilic groups, it being understood that the cation generated by their removal is a carbonium cation.

It is not essential to the theory to assume that a carbonium cation is *actually liberated*. Indeed, as we shall see later, it would be incorrect to do so. The essential point is that scission takes place between the ester oxygen and the alcohol carbon in sulfonic esters but not in carboxylic esters.

Pursuing this idea further, it is seen that anhydride formation in the sugars resolves itself into an exchange of anions on a carbonium cation, i. e., a nucleophilic displacement on carbon. The anion displaced may be halogen, sulfonic ion, the ion of a mineral acid, in other words a cationogen, while the displacing nucleophilic group is provided by a hydroxyl present in the sugar molecule. The action of sodium methoxide on a sugar *p*-toluenesulfonate is thus represented:



(Throughout this review electron transfer is indicated by curved arrows and an unshared electron pair by two dots. Ts = tosyl = *p*-toluenesulfonyl.)

The displacement is effected preferentially by a nucleophilic group already in the molecule. Thus, in the above reaction there is no evidence that OTs<sup>-</sup> is displaced by the external ion, OMe<sup>-</sup>, under the conditions used, nor is there any displacement of OTs<sup>-</sup> of a sugar sulfonate by OAc<sup>-</sup>, such as was found to occur when the sulfonate of a monohydric aliphatic alcohol was treated with potassium acetate.<sup>11</sup>

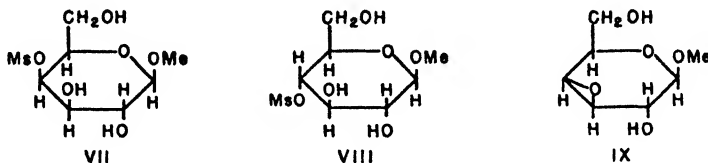
In order that anhydro ring closure should occur, it is necessary that a second hydroxyl group be available in the molecule. If all the hy-

(11) S. Peat and L. F. Wiggins, *J. Chem. Soc.*, 1088 (1938).

droxyl groups in a sugar halide or sulfonate are protected by substitution, is it possible to saponify the ester and replace the halogen atom or the tosyl group by an external anion? The answer to this question is that a number of cases of such exchange are known but the exchange is, as a rule, notoriously difficult to effect. Ease of hydrolysis appears to require the concomitant establishment of an anhydro ring.

### 1. *Trans-Exchange*

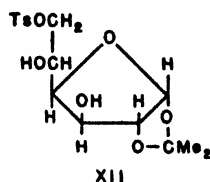
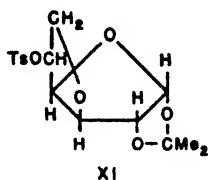
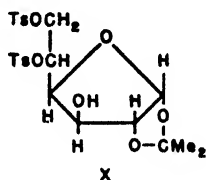
It does not follow, because one or more free hydroxyl groups are available in a sugar sulfonate, that saponification will invariably yield an anhydro sugar. On the contrary, it is very clear that the displacing ion must have a definite orientation with regard to the displaced ion before reaction resulting in anhydride formation can occur. In the first place, anhydro ring closure is most readily effected if the exchanging nucleophilic groups are on adjacent carbon atoms so that an ethylene oxide ring results. In the second place, these adjacent anions exchanging on the carbonium cation must originally be *trans*-situated with respect to each other. This is strikingly illustrated<sup>12</sup> by a comparison of the action of sodium methoxide on methyl 4-mesyl- $\beta$ -D-galactopyranoside (VII) and methyl 4-mesyl- $\beta$ -D-glucopyranoside (VIII).



(Ms = mesyl = methanesulfonyl)

The D-glucoside VIII, in which the exchanging anions on C3 and C4 are *trans*-situated, is rapidly transformed into methyl 3,4-anhydro- $\beta$ -D-galactopyranoside (IX), but sodium methoxide is without effect on the D-galactoside VII, either in the removal of the mesyl group or in dehydration. There are just as many hydroxyl groups available in VII as in VIII but in the D-galactoside the hydroxyl fixed in the ring on the adjacent carbon atom C3 is orientated *cis* with respect to the mesyl group and therefore no displacement occurs. It is to be noted that there is apparently no tendency in the D-galactoside VII for a 2,4-anhydro ring to be formed although the hydroxyl on C2 is in the *trans*-position. There is likewise no tendency for reaction to occur with the freely rotatable hydroxyl on C6, or for the mesyl group to be replaced by the external methoxyl ion.

The preceding investigation provides further evidence that the four-membered anhydro ring is not a favored form, since neither 2,4-anhydro nor 4,6-anhydro ring closure occurs in the methyl  $\beta$ -D-glucoside (VIII). On the other hand, the hydrofuranol ring is easily formed if the opportunity for the preferential establishment of an ethylene oxide ring is not offered, i. e., if a free *trans*-situated hydroxyl group is not present on an adjacent carbon atom. This condition obtains, for example, in methyl 6-bromo-D-glucopyranoside which gives methyl 3,6-anhydro-D-glucopyranoside,<sup>3</sup> and in 5,6-ditosyl-1,2-isopropylidene-D-glucofuranose (X) which yields 3,6-anhydro-5-tosyl-isopropylidene-D-glucofuranose (XI).<sup>4</sup> The 5-tosyl group may be removed from XI only by prolonged treatment with alkali.



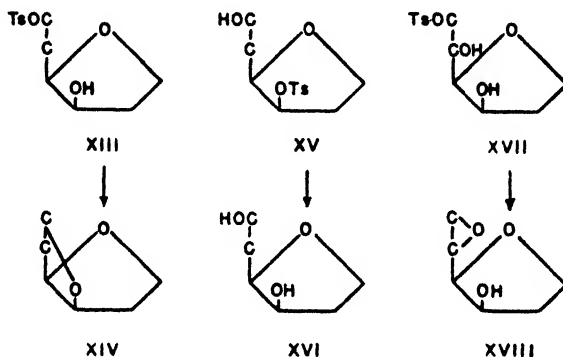
If the disposition of hydroxyl groups is such that either an ethylene oxide or a hydrofuranol ring could be formed, then it is the three-membered anhydro ring that is preferentially established. Thus, if 6-tosyl-isopropylidene-D-glucofuranose (XII), in which there are present free hydroxyls at C3 and C5, is submitted to alkaline hydrolysis, it is the 5,6-anhydride VI alone that is formed<sup>7</sup>; the 3,6-anhydride appears only if the hydroxyl at C5 is protected by substitution as in X.

Superficially it would seem that the mechanism of *trans*-elimination is operative only in the formation of anhydrides of the ethylene oxide type and that hydrofuranol ring closure takes place between hydroxyl groups which are not *trans*-disposed with regard to the sugar ring. The formation of 3,6-anhydro-D-glucose,<sup>3</sup> 3,6-anhydro-D-galactose<sup>13</sup> and 3,6-anhydro-D-mannose<sup>14</sup> from the corresponding 6-bromo or 6-tosyl compounds are examples of *cis*-elimination. It is to be observed, however, that C6 is neither a member of the sugar ring nor is it asymmetric. The cases of three-membered and five-membered anhydro ring formation are therefore not strictly comparable. It is significant in this connection that, although the transformation of 5,6-ditosyl-1,2-isopropylidene-D-glucofuranose (X) into the 3,6-anhydro derivative XI is smoothly effected, ring closure does not take place if the positions of the tosyl and hydroxyl are reversed, i. e., if the tosyl group is on C3 and the free

(13) H. Ohle and H. Thiel, *Ber.*, **66B**, 525 (1933).

(14) F. Valentin, *Collection Czechoslov. Chem. Commun.*, **6**, 354 (1934).

hydroxyl group on C6. Thus, saponification of 3-tosyl-isopropylidene-D-glucufuranose proceeds with extreme slowness, the product is that of normal ester hydrolysis, namely, isopropylidene-D-glucufuranose, and no hydrofuranol ring is formed.<sup>15, 11</sup> Reference will be made later to this surprising difference in reactivity which is illustrated diagrammatically as follows:



## 2. Inversion of Configuration

Let us now examine another consequence of the hypothesis that anhydro ring formation in the sugars is effected mainly by a process of *trans*-exchange of anionoid groups on a potential carbonium cation. It is now generally accepted as highly probable that the inversion of configuration observed in so many reactions originates in just such an anion displacement on carbon. If the carbonium cation derives from an asymmetric atom, then the configuration change becomes manifest in an inversion of optical rotation (Walden inversion). When optical inversion is observed, it is clear that the displacing anion must occupy a position, *relative to the asymmetric carbon atom*, which is sterically *opposite* to that previously occupied by the displaced group. It is therefore not unreasonable to suppose that when inversion of configuration occurs, the entering group approaches the asymmetric center from the side opposite to that where the group ultimately displaced is located. This will be true equally for intermolecular and intramolecular reaction. Sugar anhydride formation is an intramolecular reaction and as the exchange is effected by *trans*-disposed groups, ring closure should be accompanied by inversion of configuration of the carbon atom which originally bore the displaced anion. This has been verified for all cases where configuration change could be detected by the observation of optical inversion, i. e., for all cases in which the cationogenic group (halogen,

(15) H. Ohle and H. Wilcke [with, in part, K. Tessmar], *Ber.*, 71B, 2316 (1938).

tosyl, etc.) was attached to an asymmetric carbon. It is not possible, of course, to detect change of configuration in a sugar on the terminal carbon atom (C6 of a hexose) for it is not asymmetric. It is well to note that it is the carbon atom bearing the halogen or tosyl group that suffers inversion, and not the atom carrying the displacing anionoid oxygen. Thus, the 5,6-anhydro hexose prepared from 6-bromo-D-glucose is a derivative of D-glucose and not of L-idose. It would be a derivative of the latter sugar if inversion on C5 accompanied anhydride formation.

Anhydride formation by *trans*-exchange of anions on the one hand, and inversion of configuration on the other are obviously interdependent phenomena and, furthermore, an essential condition for anhydro ring formation is the *trans*-disposition of the exchanging groups. At first sight, it would appear that the formation of 3,6-anhydro rings by the saponification of 6-tosyl or 6-bromo hexoses contradicts this principle. But the contradiction is not real. It is to be remembered that the "opposite side approach" refers to the carbonium cation on which the exchange ultimately takes place and not to the sugar ring. In the case illustrated by XIII  $\rightarrow$  XIV, the exchange takes place on the carbon atom carrying the tosyl group. This carbon atom is not a member of the sugar ring, and free rotation about any of its bonds is possible. In the establishment of the 3,6-anhydro ring, rotation about the C5-C6 bond will allow the tosyl group to assume a position in which it is furthest removed from the anionoid oxygen (on C3), the position of the latter with respect to the carbon atom 6 being fixed, of course, by the exigencies of the cyclic structure. Consequently, *trans*-exchange is quite possible in the formation of 3,6-anhydro rings when the exchange takes place on C6. The state of affairs is quite different when the anion exchange is presumed to take place not on C6 but on C3. In this case, the position of the tosyl group is fixed in space (see XV) and the anionoid oxygen on C6 cannot, by virtue of free rotation, ever be brought into a position in which it and the tosyl group are on the opposite sides of C3. The tosyl group on C3 therefore is not displaced by the anionoid oxygen on C6 and the 3,6-anhydride is not formed. We have seen that the action of  $\text{Na}^+\text{OH}^-$  on XV is a normal replacement of  $\text{OTs}^-$  by  $\text{OH}^-$ . This is an intermolecular reaction which proceeds reluctantly and occasionally not at all. Although the exchanging anions are, in both cases, tosylate ion and anionoid oxygen, the mechanism is clearly different inasmuch as there is no inversion of configuration on C3 in the intermolecular reaction, whereas the intramolecular reaction probably involves, on the above argument, configurational inversion on C6.

One further point. In all the cases of anhydride formation discussed, the displacing ion has been anionoid oxygen derived from a hydroxyl

group by proton removal. If this is the invariable course of the reaction, it is clear that hydrolysis resulting in dehydration can only be accomplished by alkaline reagents. This is supported by experiment, and so far as the reviewer is aware, there is no example known of the hydrolysis of a sugar ester by acids which has led to anhydride formation.

#### IV. THE SCISSION OF ANHYDRO RINGS OF THE ETHYLENE OXIDE TYPE

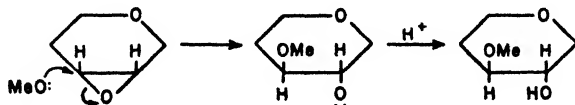
The different types of sugar anhydrides show widely different stability toward hydrolytic reagents. Whereas the ethylene oxide type undergoes ring scission with either acid or alkali, the normal hydrofuranol ring is resistant to both. The glycosans, even the five-membered ring glycosans, while being stable to alkali, are easily hydrated in the presence of acid and the ring opening in this case is not attended by Walden inversion on any save the glycosidic carbon (C1).<sup>16</sup>

Most investigations of the mechanism of ring scission have been made by studying the action of alkali on ethylene oxide anhydro sugars. These investigations will now be reviewed.

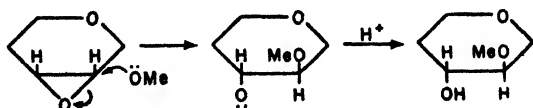
##### 1. Alkaline Scission

In the saponification of sugar sulfonic esters, it is customary to use sodium methoxide in methanol as the saponifying agent. The same reagent is also conveniently used, under more drastic conditions of temperature, for the scission of the ethylene oxide anhydro rings so formed. The scission of an anhydro ring, like its closure, may be considered in terms of the exchange of anions on a carbonium cation. The scission, however, is an intermolecular reaction and the displacing anion is methoxyl ion. The exchange may be represented, in generalized form, as follows:

Scheme A :



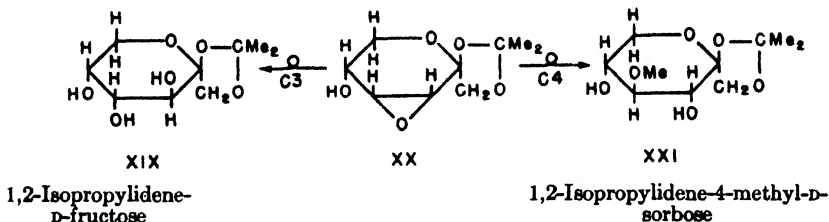
Scheme B :



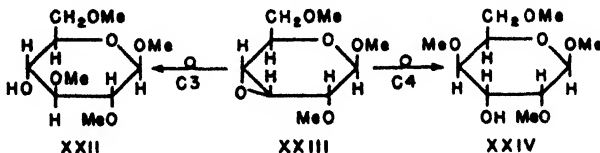
(16) K. Freudenberg and E. Braun, *Ann.*, 460, 288 (1928).

It is obvious that (1) there are two carbon centers at which the anion exchange might occur, (2) inversion of configuration will occur only on that carbon atom which develops cation reactivity, (3) the carbon at which inversion occurred is "labeled" by the displacing anion ( $\text{OMe}^-$ ) becoming attached to it. Two configurationally different sugars are therefore produced by the ring scission of an anhydro sugar and the latter is itself different in configuration from either. The relative proportion of the two sugars formed is dependent upon the configuration of the anhydro sugar as a whole, on the substituent groups in it and upon the nature of the reagent used.

Some examples will illustrate the applicability of this generalization in so far as it concerns alkaline scission. 5,6-Anhydro-1,2-isopropylidene-D-glucufuranose with alcoholic sodium hydroxide gives a mixture of isopropylidene-D-glucose and isopropylidene-L-idose. The latter results from inversion on C5, the former presumably by inversion on the non-symmetric C6.<sup>7</sup> 3,4-Anhydro-1,2-isopropylidene-D-psicose (or allulose<sup>17</sup>) (XX) when treated with sodium hydroxide yields a mixture of products among which 1,2-isopropylidene-D-fructose (XIX) was detected (in the representations inversions are denoted by circles above the arrows and the carbons inverted are noted below the arrows). With sodium methoxide, however, 1,2-isopropylidene-4-methyl-D-sorbose (XXI) is the chief product and results from inversion on C4.<sup>18</sup>



With sodium methoxide, methyl 2,6-dimethyl-3,4-anhydro- $\beta$ -D-alloside (XXIII) gives a mixture of methyl 2,3,6-trimethyl- $\beta$ -D-glucoside (XXII) and methyl 2,4,6-trimethyl- $\beta$ -D-guloside. This result provided the first



(17) F. W. Zerban and L. Sattler, *J. Am. Chem. Soc.*, **64**, 1740 (1942).

(18) H. Ohle and F. Just, *Ber.*, **68B**, 601 (1935).

clear evidence that one and the same alkaline reagent effected the ring opening in each of the two possible directions indicated by schemes A and B above (Peat and Wiggins<sup>11</sup>). In the case of methyl 2,3-anhydro-dimethyl- $\beta$ -D-mannopyranoside, the products of scission with sodium methoxide, namely, methyl 2,4,6-trimethyl- $\beta$ -D-glucopyranoside and methyl 3,4,6-trimethyl- $\beta$ -D-altroside, were produced in equi-molecular proportions, indicating equal facility of cleavage on each side of the oxygen of the anhydro ring.<sup>19</sup> When dry ammonia was used, however, instead of sodium methoxide as cleavage agent, the proportion of the two amino-sugars produced was not equimolecular; the amino-D-glucose represented only 10% of the amino-D-glucose, amino-D-altrose mixture<sup>20</sup> (see pages 167 and 168).

In many earlier examples, the isolation of only one of the two possible products of alkaline cleavage has been recorded. It is not clear whether this is due to the omission of a search for a second constituent or whether, in some cases, the scission is 100% in one direction.<sup>21</sup>

## 2. Scission by Acids

It is possible to regard the transformation of an anhydro sugar into an amino sugar as an example of acid fission of the anhydro ring, inasmuch as the active exchanging anion is  $\text{NH}_2^-$  derived from ammonia (which is used in dry ether or alcohol) thus:  $\text{NH}_3 \rightleftharpoons \text{H}^+ + \text{NH}_2^-$ . If this is in fact an example of acid fission, then the same constitutional and configurational changes are induced as by alkaline scission. The data available are, however, inadequate at present to enable conclusions to be drawn concerning fission by acids comparable to the conclusions reached about the course of alkaline fission. The examples of ring cleavage by acids recorded in the literature indicate that the mechanism of the reaction probably does not differ in essentials from that of alkaline scission, already detailed. Thus, aqueous sulfuric acid reacting with methyl 3,4-anhydro- $\beta$ -D-galactoside gave a mixture of sugars in which D-glucose and D-gulose but no D-galactose were identified.<sup>22</sup> When the same methyl anhydro-D-galactoside is treated with hydrochloric acid, the introduction of a chlorine atom accompanies ring scission.<sup>23</sup> Presumably the chlorine anion functions here in the same capacity as does the methoxyl ion when sodium methoxide is the hydrolyzing agent. The anhydro rings of methyl 2,3-anhydro-dimethyl-D-alloside and methyl

(19) W. H. G. Lake and S. Peat, *J. Chem. Soc.*, 1417 (1938).

(20) W. N. Haworth, W. H. G. Lake and S. Peat, *J. Chem. Soc.*, 271 (1939).

(21) G. J. Robertson, W. H. Myers and W. E. Tetlow, *Nature*, 142, 1076 (1938).

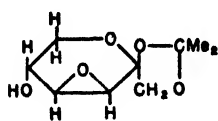
(22) A. Müller, *Ber.*, 68B, 1094 (1935).

(23) A. Müller, *Ber.*, 67B, 421 (1934).

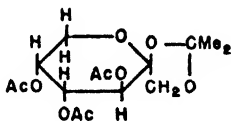


3,4-anhydro-dimethyl-D-alloside are also opened by hydrochloric acid. In each case two chlorodimethylhexoses are produced and these are not identical.<sup>11</sup> A little earlier it had been shown that two methyl chlorohexosides were produced simultaneously when methyl benzyldiene-2,3-anhydro-D-alloside was treated with very dilute acetone-hydrogen chloride (nearly anhydrous). Each of these chloro compounds yielded the same methyl 2,3-anhydro-D-alloside when treated with silver oxide.<sup>24</sup>

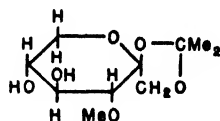
An interesting contrast has been observed between the respective actions of acid and alkali on 3,4-anhydro-1,2-isopropylidene-D-tagatose (XXIVa). The anhydro ring of this compound shows considerable resistance to both mineral acids and alkali. Cleavage by sodium meth-



XXIVa



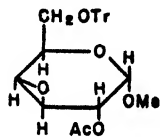
XXV



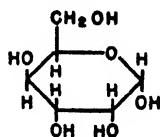
XXVI

oxide is accompanied by inversion on C3 and 1,2-isopropylidene-3-methyl-D-sorbose (XXVI) results. When, however, cleavage is effected by means of an acetic acid, acetic anhydride mixture containing a little pyridine, inversion takes place predominantly not on C3 but on C4 and the D-fructose derivative (XXV) is obtained in more than 50% yield.<sup>25</sup>

None of the examples just quoted can be regarded as in disagreement with the hypothesis that alkali and acid scissions of anhydro rings are accomplished by the same essential mechanism. A discordant note is however struck by the observation<sup>26</sup> that D-galactose is obtainable from the mixture which results when the 3,4-anhydro-D-galactose derivative (XXVII) is treated in the cold with dry hydrogen chloride in acetone. The presence of D-gulose (XXVIII) was also indicated and this could arise in normal fashion by inversion on C3. D-Galactose, however,



XXVII



XXVIII

D-Gulose

Tr = trityl =  $(C_6H_5)_3C-$

(24) G. J. Robertson and H. G. Dunlop, *J. Chem. Soc.*, 472 (1938).

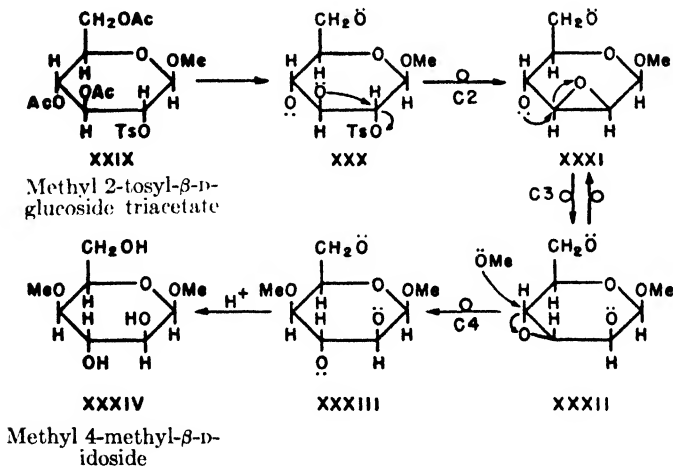
(25) H. Ohle and C. A. Schultz, *Ber.*, 71B, 2302 (1938).

(26) J. W. H. Oldham and G. J. Robertson, *J. Chem. Soc.*, 685 (1935).

could be produced only if cleavage occurred without inversion of configuration on either C3 or C4. This is the only example known to the reviewer which does not conform to the view that the mechanism of ethylene oxide ring scission invariably involves the *trans*-exchange of anionoid groups.

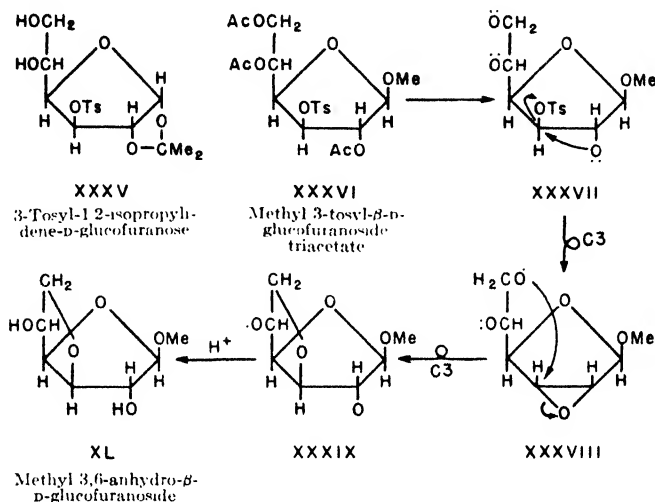
### 3. Anhydro Sugars as Intermediates in the Interconversion of Configurational Isomers

Although the conditions required for alkaline scission of three-membered anhydro rings are, in general, more drastic than the conditions for ring closure, nevertheless there is strong evidence that, in many cases, ring opening and ring closure can occur as simultaneous reactions. It is otherwise not possible to explain the conversion by alkaline hydrolysis of methyl 2-tosyl-D-glucoside into a derivative of D-idose (Lake and Peat<sup>27</sup>). The conversion of D-glucose into D-idose required that inversion of configuration should occur on C2, C3 and C4. Such inversion would be the natural consequence of the intermediate formation and fission of a 2,3-anhydro ring followed by that of a 3,4-ring. The action of Na<sup>+</sup>OMe<sup>-</sup> on methyl 2-tosyl-β-D-glucoside triacetate (XXIX) could be represented by the sequence XXIX to XXXIV.



Another example is provided by the observation of Ohle and Wilcke<sup>16</sup> that whereas 3-tosyl-1,2-isopropylidene-D-glucofuranose (XXXV) is saponified without anhydro ring formation and without Walden inversion, the triacetate of methyl 3-tosyl-β-D-glucofuranoside (XXXVI) is

rapidly converted by alkali into methyl 3,6-anhydro- $\beta$ -D-glucofuranoside (XL). The significant structural difference between XXXV and XXXVI is that in the latter, the hydroxyl group on C2 is not protected against alkali and can thus function as an exchanging anion *trans*-situated with respect to the tosyl group on C3.

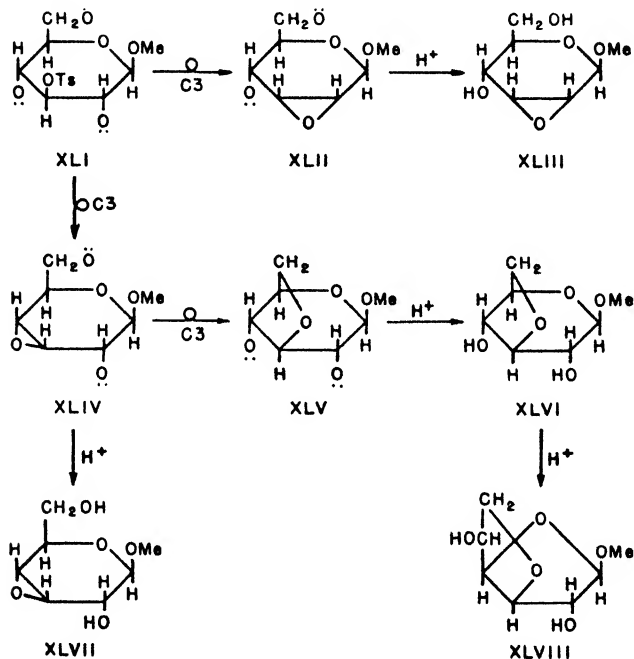


The inference is that the hydrofuranol ring of XL can never be directly formed by the saponification of a 3-tosyl ester of D-glucose, but only indirectly by the intermediate formation and scission of an anhydro ring of the ethylene oxide type. The sequence of reactions involved in the conversion of methyl 3-tosyl- $\beta$ -D-glucoside into methyl 3,6-anhydro- $\beta$ -D-glucoside is shown by XXXVI to XL.

This conclusion lends the strongest support to the hypothesis that anhydro ring formation is conditioned by, and is a consequence of, the exchange on a potential carbonium cation of anions which are *trans*-disposed with respect to the cation. For instance, in XXXV, the anions on C3 and C6 which might exchange are *cis*-oriented with regard to the carbonium ion C3 and therefore no exchange takes place and no 3,6-anhydro ring is formed. In the allose derivative (XXXVIII), however, the groups on C6 and C3 are *trans*-disposed and the hydrofuranol ring can now form, giving XXXIX which again has the configuration of D-glucose.

Another example of the formation of a 3,6-anhydro ring by the saponification of a 3-tosyl-D-glucose derivative is furnished by the work of Peat and Wiggins<sup>11</sup> who were able to isolate derivatives of no less than three anhydro sugars, one of which was 3,6-anhydro-D-glucose, from the

saponification of methyl 3-tosyl- $\beta$ -D-glucopyranoside. Two of the products were of the ethylene oxide type, namely, methyl 2,3-anhydro- $\beta$ -D-alloside (XLIII) and methyl 3,4-anhydro- $\beta$ -D-alloside (XLVII). Having regard to the observations described above, Ohle and coworkers<sup>15</sup> made the reasonable suggestion that here also the methyl 3,6-anhydro-D-glucoside was not directly produced but resulted from the cleavage of the methyl 3,4-anhydro-D-alloside which was the true primary product. The sequence of events is shown by the formulas XLIV to XLVIII.



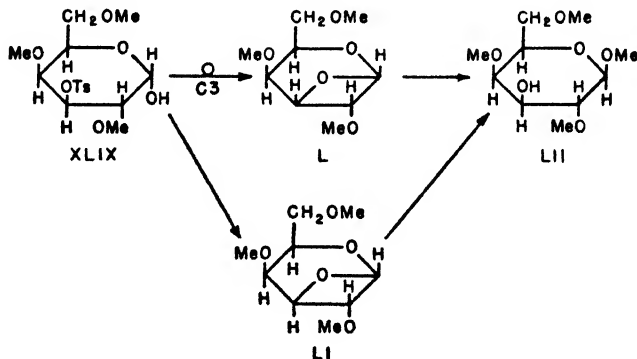
When this paper<sup>11</sup> was published (in 1938), the evidence indicated that the hydrofuranol ring compound isolated was the pyranoside form (XLVI). The later work of Haworth, Owen, and Smith,<sup>28</sup> which is discussed in section V-1, corrected this impression and proved that the compound actually isolated by Peat and Wiggins was methyl 3,6-anhydro- $\beta$ -D-glucofuranoside (XLVIII). This correction does not invalidate the above argument, for it is known that XLVI is unstable and a trace of acid suffices to transform it into the stable furanoside.

*a. The Interconversion of Sugars in Nature.* It is pertinent now to inquire what part, if any, anhydro sugar formation plays in the metabolic transformations of sugars in plants and animals. One of the most

striking of these transformations is that which takes place with such smooth elegance in the mammary gland, namely the conversion of D-glucose into D-galactose. The mechanism of this conversion has intrigued chemists for years. Superficially it is simple, being merely an inversion of configuration on C4, but it has never been successfully accomplished in the laboratory except in the case described by Oldham and Robertson,<sup>26</sup> the exceptional nature of which has been commented upon in section IV-2 of this review. Before the mechanism of hydrolysis of sugar esters had been fully elucidated, the suggestion was made that the inversion on C4 was possibly effected by the saponification of D-glucose 4-phosphate. We now know however that such hydrolysis would lead in the first place to an anhydro-D-galactose and that alkaline scission of the ring of the latter would not be expected to give D-galactose.

Anhydro sugars of the ethylene oxide type have never been detected as constituents of any living material but this fact does not necessarily prove that anion exchange on carbonium ion is not involved in the biological interconversion of sugars. It is possible, for instance, to suggest a mechanism for the conversion of D-glucose into D-galactose in terms of such anion exchange. The mineral acid most likely to function biologically in the esterification of D-glucose is phosphoric acid, and the hypothesis requires the formation of D-glucose 5-phosphate and subsequent intramolecular anion exchange on C5 and C4, successively.

*b. Some Exceptional Cases.* Examples are known of configurational change in the sugar series in which it is inferred that anhydro ring formation is an intermediate step although no anhydro sugar can be isolated. Thus Percival and Percival<sup>29</sup> commented on the fact that whereas methyl 3-tosyl-trimethyl-D-galactopyranoside is extremely resistant to the action of sodium methoxide, the corresponding sugar, 3-tosyl-trimethyl-D-galactopyranose (XLIX), undergoes saponification with ease.



The product retains, however, the configuration of D-galactose and is in fact methyl trimethyl-D-galactopyranoside (LII). The intermediate formation of a 1,3-anhydro ring is postulated.

If this is indeed the course of the saponification, then it represents an exception to the general hypothesis of anion exchange and inversion. The 1,3-anhydro compound will have the configuration of D-glucose (L) if normal inversion on C3 accompanies saponification. Ring scission of L could yield LII only if accompanied by inversion on C3. The displacing anion ( $\text{OMe}^-$ ) becomes attached, however, not to C3 but to C1, an indication that ring scission has occurred rather on the C1 side of the bridge oxygen. The alternative explanation is that saponification and 1,3-ring formation takes place without inversion on C3 and that the intermediate 1,3-anhydro compound (LI) retains the configuration of D-galactose. Cleavage of the anhydro ring of LI, if this takes place entirely at the C1-oxygen linkage, would normally yield the D-galactoside (LII). It is well to bear in mind that the postulated anhydro ring is altogether exceptional in being both four-membered and glycosan in type.

A curious observation made by Levene and Compton<sup>30, 31</sup> is connected with the subject of this section. The saponification of 5-tosyl-2,3-isopropylidene-L-rhamnofuranose (L-rhamnose = 6-desoxy-L-mannose) with sodium methoxide takes place in the cold and the product is methyl 2,3-isopropylidene-6-desoxy-D-allofuranoside. Inversion occurs therefore not only on C5 but also on C4, and moreover C1 is also involved inasmuch as the displacing anion ( $\text{OMe}^-$ ) is ultimately located there in the product. The authors suggest that the reaction proceeds by virtue of the intermediate formation and scission of a 4,5-anhydro and a 1,5-anhydro compound, the former presumably having the configuration of D-glucose, the latter that of D-allose.

Yet another example of this phenomenon is equally difficult to reconcile with the general hypothesis. When 4-tosyl-2,3,6-trimethyl-D-glucose is treated with sodium methoxide, a non-reducing anhydro trimethyl hexose results (Hess and Neumann<sup>32</sup>). Hydrolysis of the anhydride with dilute hydrochloric acid yields 2,3,6-trimethyl-L-idose. The isolation of a derivative of L-idose indicates that removal of the tosyl group on C4 is apparently not attended by inversion on that carbon atom. Instead, inversion occurs on C5. It is possible that here again, multiple formation and scission of anhydro rings is occurring. The fact that the anhydride does not reduce Fehling's solution and is easily hydrolyzed by aqueous acid shows that it is of the glycosan type and could be de-

(30) P. A. Levene and J. Compton, *J. Am. Chem. Soc.*, **57**, 2306 (1935).

(31) P. A. Levene and J. Compton, *J. Biol. Chem.*, **116**, 169 (1936).

(32) K. Hess and F. Neumann, *Ber.*, **68B**, 1360 (1935).

scribed as a 1,4-anhydro 2,3,6-trimethyl hexose. It very probably has the same configuration as the sugar produced from it when the internal glycosidic group (glycosan link) is hydrolyzed by acid, i. e., the configuration of L-idose. It certainly has not the configuration of D-glucose if it be accepted that the product of the action of sodium on 1-chloro-2,3,6-trimethyl-D-glucose is 1,4-anhydro-2,3,6-trimethyl-D-glucopyranose.<sup>33</sup> The problem is complicated further by the isolation of a third isomeric anhydride, which is obtained by the saponification of 5-tosyl-trimethyl-D-glucufuranose (Hess and Heumann<sup>34</sup>).

## V. ANHYDRO SUGARS OF THE HYDROFURANOL TYPE

### 1. 3,6-Anhydro Hexoses

*a. Ring Closure.* The first 3,6-anhydro hexose to be prepared was obtained by the saponification of a 6-bromo derivative of D-glucose.<sup>3</sup> It is now recognized that for the establishment of the 3,6-anhydro bridge in a hexose three conditions are necessary.

- (1) The terminal primary alcohol group must carry a cationogenic substituent, i. e., a substituent the removal of which by alkali takes place in such a way that C6 becomes a carbonium cation.
- (2) The fifth carbon atom (C5) must not carry a free hydroxyl group.
- (3) A free hydroxyl group must be available (or become available under alkaline conditions) on C3 and this hydroxyl group must (for steric reasons) be on the same side of the sugar ring as the side-chain, C6.

This procedure provided the most important method for the synthesis of anhydro sugars of the hydrofuranol type. 3,6-Anhydro sugars have resulted from the saponification of hexoses esterified at C6 by the following cationogens:  $\text{Br}^-$  <sup>3, 35, 14, 36</sup>;  $\text{CH}_3\cdot\text{C}_6\text{H}_4\cdot\text{SO}_2\text{O}^-$  <sup>5, 37, 38, 28, 39, 40</sup>;  $\text{HO}\cdot\text{SO}_2\cdot\text{O}^-$  <sup>41, 42</sup>;  $\text{O}_2\text{N}\cdot\text{O}^-$  <sup>43</sup>. It is very probable that the phosphate ion is also cationogenic and that saponification of a hexose 6-phosphate

(33) K. Freudenberg and E. Braun, *Ann.*, **460**, 288 (1928); *Ber.*, **66B**, 780 (1933).

(34) K. Hess and K. E. Heumann, *Ber.*, **72B**, 137 (1939).

(35) E. Fischer and K. Zach, *Ber.*, **45**, 2058 (1912).

(36) F. Valentin, *Collection Czechoslov. Chem. Commun.*, **8**, 35 (1936).

(37) H. Ohle and R. Lichtenstein, *Ber.*, **63B**, 2905 (1930).

(38) W. N. Haworth, J. Jackson and F. Smith, *J. Chem. Soc.*, 620 (1940).

(39) T. S. Gardner and C. B. Purves, *J. Am. Chem. Soc.*, **65**, 444 (1943).

(40) (Mrs.) P. A. Rao and F. Smith, *J. Chem. Soc.*, 229 (1944).

(41) E. G. V. Percival and T. H. Soutar, *J. Chem. Soc.*, 1475 (1940).

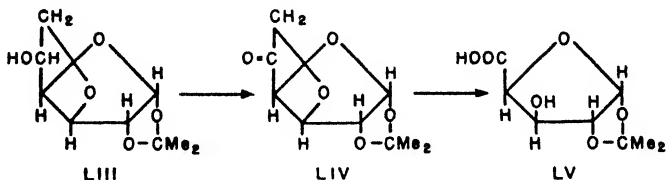
(42) R. B. Duff and E. G. V. Percival, *J. Chem. Soc.*, 830 (1941).

(43) E. K. Gladding and C. B. Purves, *J. Am. Chem. Soc.*, **66**, 76, 153 (1944).

of suitable steric form would lead to a 3,6-anhydro hexose. It is indeed known that anhydro ring formation does result from the hydrolysis of sugar phosphoric esters when the phosphate group occupies position 3,<sup>44, 46</sup> such hydrolysis being, of course, attended by inversion on C3. Percival and Percival<sup>45a</sup> have now shown that certain non-reducing derivatives of D-glucose 3- and 6-phosphates undergo saponification with alkali. The scission of the phosphoric ester link was not accompanied, however, either by Walden inversion or by anhydride formation. The second preparative procedure, the saponification of sugar esters in which the cationogenic group is on C3, has already been discussed in section III-1.

A third method which has been used only sparingly hitherto consists in the deamination of a 3-amino hexose<sup>46</sup> or of a 6-amino hexose.<sup>47</sup>

*b. Proof of Constitution.* The distinguishing mark of the hydrofuranol ring is its stability toward both acid and alkaline hydrolyzing agents and also toward many oxidizing agents. Establishment of the configuration of the anhydro sugar is therefore a problem of great difficulty and, indeed, the originally tentative assignment of the D-glucose configuration to the 3,6-anhydro hexose prepared from 6-bromo-D-glucose has been tacitly accepted for many years without experimental proof. An approach to a solution of the problem was indicated by Ohle and Erlbach in 1929.<sup>47</sup> It was found that 3,6-anhydro-1,2-isopropylidene-D-glucofuranose (LIII) slowly decolorized neutral permanganate solution and from the oxidation products was isolated an acid having the composition and properties of 1,2-isopropylidene-D-xyluronic acid (LV). The course of the oxidation may be represented by LIII to LV.



The configuration of the penturonic acid LV was not however conclusively proved to be that of D-xylose and consequently this experiment is not determinative in regard to the configuration, as distinct from constitution, of 3,6-anhydro-D-glucose.

(44) P. A. Levene, A. L. Raymond and A. Walti, *J. Biol. Chem.*, **82**, 191 (1929).

(45) A. L. Raymond and P. A. Levene, *J. Biol. Chem.*, **83**, 619 (1929).

(45a) E. E. Percival and E. G. V. Percival, *J. Chem. Soc.*, 874 (1945).

(46) P. A. Levene and H. Sobotka, *J. Biol. Chem.*, **71**, 181 (1926).

(47) H. Ohle and H. Erlbach, *Ber.*, **62B**, 2758 (1929).

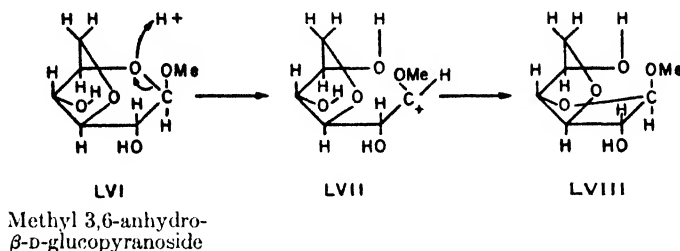


*c. The Stability of the Dicyclic System.* Methyl 3,6-anhydro glycosides are dicyclic structures and are of two types: (a) those having the hydrofuranol ring associated with the six-membered (or pyranose) sugar ring; and (b) those having two five-membered rings (the hydrofuranol and the furanose) in the same molecule. In simple glycosides, the pyranoside form is relatively much more stable toward acid hydrolyzing agents than is the furanoside but the introduction of an oxygen bridge between C3 and C6 reverses this order of stability. For example methyl 3,6-anhydro- $\beta$ -D-glucopyranoside (LVI) can only be obtained if the pyranose ring is stabilized by methyl glycoside formation *before* the hydrofuranol ring is established. If anhydro ring closure precedes glycoside formation, as it does in the treatment of 3,6-anhydro-D-glucose with methyl alcoholic hydrogen chloride, the methyl pyranoside is not formed. Instead, the much more stable methyl 3,6-anhydro- $\beta$ -D-glucofuranoside (LVIII) is obtained. The stability of these dicyclic systems has been investigated by Haworth, Smith and coworkers<sup>38, 28</sup> who conclude that the hydrofuranol ring assumes the character of the principal ring to which the sugar ring (pyranose or furanose) is subsidiary, and that the unusual properties of 3,6-anhydro sugars and their derivatives are dependent upon that fact. It is appropriate to mention at this stage that Cottrell and Percival<sup>48</sup> have recently made the observation that the 3,6-anhydro ring is disrupted with the greatest of ease under certain conditions. When methyl 3,6-anhydro- $\beta$ -D-galactopyranoside was submitted to acetolysis, among the products isolated was *aldehydo*-D,L-galactose heptaacetate.

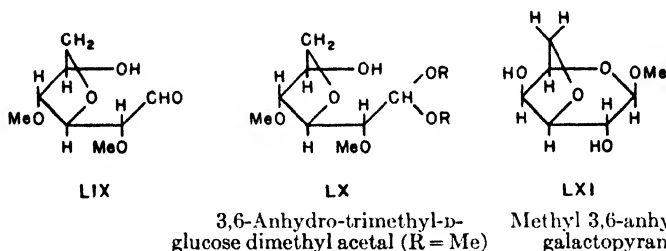
It is usually postulated that, in the interconversion of the anomeric methyl glycosides or of methyl furanoside and methyl pyranoside, a necessary intermediate stage is the liberation of the free sugar by the hydrolytic removal of the glycosidic methyl group, but a remarkable outcome of the work of Haworth, Smith and coworkers is the demonstration that methyl 3,6-anhydro-D-glucopyranoside is directly converted into the corresponding furanoside without the loss of the glycosidic methyl group. The activating agent is  $H^+$  and the presence of methyl alcohol is not necessary for the conversion. Thus, the change is effected by dry hydrogen chloride, by a solution of the gas in ether and even by dilute aqueous sulfuric acid. The conclusion is inescapable that during the ring change from pyranose to furanose the glycosidic methoxyl group remains attached to C1 and it is reasonable to suppose that the proton attack is directed to the oxygen of the sugar ring. The intermediate formation is thus implied of the ion (LVII) in which C1 is represented as a carbonium cation.

(48) T. L. Cottrell and E. G. V. Percival, *J. Chem. Soc.*, 749 (1942).

When the closure of the furanose ring is not possible, as in the 2,4-dimethyl derivative of LVI (there being no longer a hydroxyl group available on C4), then non-aqueous acid simply catalyzes the interconversion of the  $\alpha$  and  $\beta$  isomers of the methyl *D*-pyranoside. The



relative proportion of the isomers produced is determined presumably by steric considerations and, in the case of methyl 2,4-dimethyl-3,6-anhydro-*D*-galactopyranoside, the equilibrium is toward the  $\beta$ -*D* isomer side. If water is present during the acid catalysis the pyranoside or furanoside ring is disrupted, the glycosidic methyl group is removed by normal hydrolysis and the resulting sugar has the properties of a free aldehyde. For instance, methyl 3,6-anhydro-2,4-dimethyl-*D*-glucopyranoside gives the open-chain or *aldehyde*-sugar LIX. If the vehicle of



the acid catalyst is an alcohol, the corresponding dialkyl acetal (LX) is produced. The influence of steric factors is well illustrated by a comparison of the action of methyl alcoholic hydrogen chloride on methyl 3,6-anhydro- $\beta$ -*D*-galactopyranoside (LXI) and its *D*-glucose equivalent (LVI), respectively. We have seen that a smooth transformation to the methyl furanoside (LVIII) takes place with LVI but with the *D*-galactose derivative (LXI), in which the configuration on C4 is the reverse of that of LVI, the furanose ring cannot be established because of the juxtaposition of the hydrofuranol ring and the hydroxyl on C4. The product is in fact the alternative strainless structure, 3,6-anhydro-*D*-galactose dimethyl acetal.

We may, perhaps, summarize this argument in the following terms.

1. A structure in which a 3,6-anhydro ring and a pyranose ring are present together is in a state of molecular strain and can be obtained only if the pyranose ring is stabilized (by glycosidic formation) *before* closure of the hydrofuranol ring. 2. The combination of a 3,6-anhydro ring and a furanose ring in glucose or mannose represents a stable, strainless system; in galactose, however, the establishment of the two rings is sterically prevented. 3. Where it is not possible for the two five-membered rings to exist together, as in galactose or in 4-methylglucose, an alternative strainless form is the open-chain *aldehyde*-form.

It is to be expected, on these grounds, that the predominating component of the mutarotation equilibrium of 3,6 anhydro-glucose will be 3,6-anhydro-glucofuranose whereas that of 3,6-anhydro-galactose will be the open-chain form. Neither will include pyranose forms.

## 2. 2,5-Anhydro Hexoses

Our present knowledge of the second type of hydrofuranol anhydro sugars, the 2,5-anhydro hexoses, is less extensive and definite than is the case with the 3,6-anhydro type. The credit for most of the information we have belongs to Levene and coworkers and results from the comprehensive study of hexosamines made by them. A good summary of this work was provided by Levene in 1921.<sup>49</sup>

The best known 2,5-anhydro hexose is that which was prepared in 1894 by Fischer and Tiemann<sup>4</sup> and named by them chitose. Chitose is a liquid with ill-defined properties and is usually characterized by its oxidation derivatives, chitonic acid and isosaccharic acid. It is prepared by the deamination of the naturally occurring chitosamine (D-glucosamine).

a. *The Constitution and Configuration of Chitose.* Chitose is readily oxidized in two stages to give an anhydro hexonic acid (chitonic acid) and an anhydro saccharic acid (isosaccharic acid), thus offering a parallel to the oxidation of D-glucose to D-gluconic acid and D-glucosaccharic acid. Isosaccharic acid was, in point of time, prepared before chitose, by the direct action of nitric acid on chitosamine<sup>50, 51</sup> but the presence in it of an anhydro ring was not initially recognized. It was considered to be an isomer of D-glucosaccharic acid, hence its name. Later, when analytically pure specimens were available, it became evident that isosaccharic

(49) P. A. Levene, *Biochem. Z.*, **24**, 39 (1921); cf. P. A. Levene, "Hexosamines and Mucoproteins," Longmans, Green and Co., London (1925).

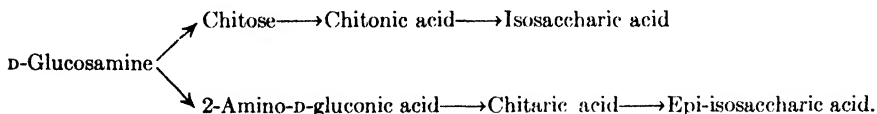
(50) F. Tiemann, *Ber.*, **17**, 246 (1884).

(51) F. Tiemann and R. Haarmann, *Ber.*, **19**, 1257 (1886).

acid was actually a dehydrated saccharic acid (a saccharic acid less one molecule of water).<sup>52</sup> When isosaccharic acid is heated above its melting point it gives furan- $\alpha$ -carboxylic acid and when heated at a lower temperature in hydrogen chloride, furan- $\alpha\alpha'$ -dicarboxylic acid. It was concluded from these observations that the anhydro ring in isosaccharic acid engages the carbon atoms C2 and C5 adjacent to the carboxyl groups.<sup>51</sup> The oxidation sequence, chitose  $\rightarrow$  chitonic acid  $\rightarrow$  isosaccharic acid, is unlikely to be accompanied by structural changes in the anhydro ring and consequently the hydrofuranol ring is assumed to be the same in all three compounds. Support for this assumption is afforded by the fact that acetylation of chitonic acid yields  $\alpha'$ -acetoxymethyl-furan- $\alpha$ -carboxylic acid.<sup>53</sup> Chitose itself is therefore a 2,5-anhydro hexose.

The problem of determining the configuration of chitose is dependent on a knowledge of the configuration of chitosamine from which it is derived. It was known that this hexosamine had the amino group on C2 and indirect evidence strongly suggested the configuration on this atom to be that of *D*-glucose. In 1939, it was conclusively proved that the amino sugar of chitin was in fact 2-amino-*D*-glucose (Haworth, Lake and Peat<sup>20</sup>). It then followed that chitose was either 2,5-anhydro-*D*-glucose or 2,5-anhydro-*D*-mannose, the first if deamination was not attended by inversion, the second if inversion occurred with the removal of the amino group. (For a discussion of the configuration of *D*-glucosamine, see pages 167 and 168.)

Let us now return to the pioneering work of Fischer and Tiemann.<sup>4</sup> Chitonic acid is produced from *D*-glucosamine by deamination and oxidation, in that order. If the order is reversed, however, i. e., if *D*-glucosamine is first oxidized to *D*-glucosaminic acid (2-amino-*D*-gluconic acid) and the latter substance subsequently deaminated, chitonic acid is not the product. Instead an isomeric 2,5-anhydro hexonic acid (chitaric acid) is obtained. These facts may be summarized thus:



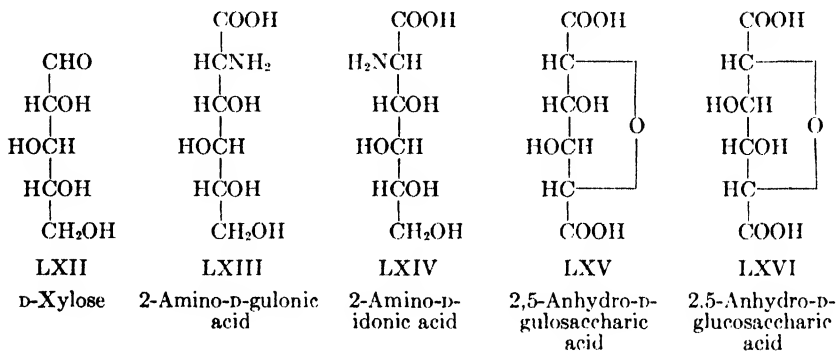
Walden inversion on C2 has occurred in one of these sequences; if in the first, then chitose, chitonic acid and isosaccharic acid have the configuration of *D*-mannose; if in the second, i. e., in the deamination of 2-amino-*D*-gluconic acid, then chitaric acid and epi-isosaccharic acid are

(52) F. Tiemann, *Ber.*, 27, 118 (1894).

(53) E. Fischer and E. Andreae, *Ber.*, 36, 2587 (1903).

D-mannose derivatives. Chitose and its oxidation products must then necessarily have a D-glucose configuration. The problem thus presented was solved by Levene and LaForge in 1915<sup>54, 55, 56</sup> in the following manner.

An application to D-xylose (LXII) of the Fischer synthesis of 2-amino hexonic acids from pentoses would lead theoretically to a mixture of 2-amino-D-gulonic acid (LXIII) and 2-amino-D-idonic acid (LXIV).



Furthermore, deamination and oxidation of the mixture of amino hexonic acids should yield 2,5-anhydro-D-gulosaccharic acid (LXV) and 2,5-anhydro-D-idosaccharic acid, and this irrespective of whether the deamination is accompanied by inversion on C2. What is important is the impossibility of synthesizing 2,5-anhydro-D-mannosaccharic acid by this procedure from D-xylose. The 2,5-anhydro-D-gulosaccharic acid (LXV) is the optical enantiomorph of 2,5-anhydro-D-glucosaccharic acid (LXVI), and Levene was able to show that one of the 2,5-anhydro saccharic acids prepared from D-xylose was indeed the enantiomorph of epi-isosaccharic acid, which, in this way, was shown to be 2,5-anhydro-D-glucosaccharic acid. It was thus clearly demonstrated that deamination of 2-amino-D-gluconic acid took place without inversion and that chitic acid and epi-isosaccharic acid retained the configuration of D-glucose. It follows that isosaccharic acid, chitonic acid and therefore chitose have the epimeric D-mannose configuration and are respectively 2,5-anhydro-D-mannosaccharic acid, 2,5-anhydro-D-mannonic acid and 2,5-anhydro-D-mannose.

*b. The Mechanism of Deamination.* In the formation of chitose (2,5-anhydro-D-mannose) from D-glucosamine (2-amino-D-glucopyranose)

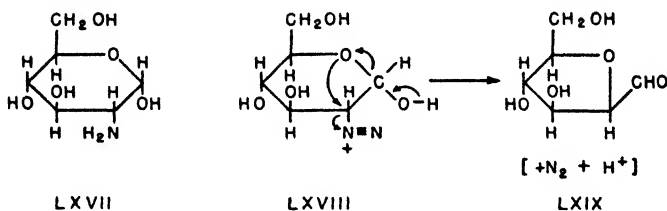
(54) P. A. Levene and F. B. LaForge, *J. Biol. Chem.*, **21**, 345 (1915).

(55) P. A. Levene and F. B. LaForge, *J. Biol. Chem.*, **21**, 351 (1915).

(56) P. A. Levene, *J. Biol. Chem.*, **36**, 89 (1918).

all the elements of anion exchange on a carbonium cation are present, with the concomitant Walden inversion and anhydro ring closure. The potential carbonium cation is C2; the cationogen is the diazotized amino group and the displacing anion is the nucleophilic oxygen of the pyranose ring. It is to be observed that the sugar ring plays a dominant part in directing the course of the reaction. If the sugar ring is absent, deamination proceeds *without* inversion, although an anhydro bridge is established. Thus, 2-amino-D-gluconic acid gives 2,5-anhydro-D-gluconic acid (chitaric acid), both compounds having the D-glucose configuration.

It would seem that the alcoholic hydroxyl group is not itself capable of functioning as the displacing nucleophilic group in an exchange on a carbonium cation leading to inversion, although it can nevertheless provide the oxygen for anhydro ring formation. Inspection of the formula of D-glucosamine (LXVII) reveals that the amino group on C2 and the hydroxyl group on C3 are *trans*-situated with respect to the cationic carbon, C2. If therefore the hydroxyl group could act as a displacing group in a *trans*-exchange, the conditions are ideal for the establishment of an ethylene oxide ring. But this does not occur, the reason presumably being that hydroxyl cannot function as a displacing group and its conversion, by loss of a proton, into anionoid oxygen, ( $>CH-OH \rightarrow >CH-O^-$ ), which can so function, does not take place in acid solution. The oxygen which actually functions as the displacing group is attached to C5, i. e., it is the oxygen atom which originally formed part of the pyranose ring. In other words, this oxygen, while remaining attached to C5, changes its allegiance from C1 to C2.



A possible electron circuit in this complex reaction is shown in LXVIII and the products of the completed reaction in LXIX. The *trans*-arrangement of the hydroxyl on C4 and the aldehyde group (C1) in LXIX makes the establishment of a furanose ring sterically unlikely and consequently 2,5-anhydro-D-mannose (chitose) probably exists as an *aldehydo*-sugar.

Further consideration needs to be given to the interesting fact that although D-glucosaminic acid (2-amino-D-gluconic acid) undergoes deamination with<sup>1</sup> the establishment of a 2,5-anhydro ring, the deamination

is not accompanied by inversion on C2. The 2,5-anhydro hexonic acid (chitaric acid) produced retains the configuration of D-glucose. The difference may be dependent partly on the fact that free rotation is possible about C2 in the open-chain D-glucosaminic acid but not in the cyclic D-glucosamine, and partly on the absence of anionoid oxygen in the case of the amino acid.

## VI. ANHYDRO SUGARS OF THE GLYCOSAN TYPE

### 1. *Preparative Methods for 1,6-Anhydro Hexoses*

*a. Dehydration by Heat.* This is usually referred to as the method of Pictet inasmuch as this author developed a technique for the preparation of levoglucosan by the vacuum distillation of D-glucose polysaccharides such as cellulose and starch<sup>57</sup> or of  $\beta$ -D-glucosides.<sup>58</sup> The most recent method using starch is given by Zemplén and Gerecs<sup>59</sup> while Karrer describes the preparation of levoglucosan from  $\beta$ -D-glucose.<sup>60</sup> A dextrorotary glucosan was prepared much earlier, in 1860.<sup>1</sup> Its preparation was improved by Pictet and Castan in 1920,<sup>61</sup> who obtained it by maintaining D-glucose at a temperature of 150° in a vacuum. It was shown to be 1,2-anhydro- $\alpha$ -D-glucopyranose.<sup>61a</sup> The method of pyrolysis has also been employed in the preparation of 1,6-anhydro- $\beta$ -D-mannopyranose from ivory nut meal<sup>62, 63</sup> and of the corresponding galactosan from agar.<sup>64</sup> The pyrolysis of lactose gives a mixture of 1,6-anhydro- $\beta$ -D-glucopyranose (levoglucosan) and 1,6-anhydro- $\beta$ -D-galactopyranose in fair yields and in fact affords the most convenient and economical source of either of these glycosans (Hann and Hudson<sup>65</sup>).

*b. Alkaline Hydrolysis of Aromatic Glycosides.* This is the method of Tanret<sup>2</sup> who prepared levoglucosan by the action of boiling baryta solution on such naturally occurring glucosides as picein, salicin or coniferin. Hudson and coworkers have recently employed this method of glycosan formation, using phenyl  $\beta$ -D-glycosides.<sup>66</sup>

(57) A. Pictet and J. Surasin, *Helv. Chim. Acta*, **1**, 87 (1918); A. Pictet and M. Cramer, *ibid.*, **3**, 640 (1920).

(58) A. Pictet and H. Goudet, *Helv. Chim. Acta*, **2**, 698 (1919).

(59) G. Zemplén and A. Gerecs, *Ber.*, **64B**, 1545 (1931).

(60) P. Karrer, *Helv. Chim. Acta*, **3**, 258 (1920).

(61) A. Pictet and P. Castan, *Helv. Chim. Acta*, **3**, 645 (1920).

(61a) M. Cramer and E. H. Cox, *Helv. Chim. Acta*, **5**, 884 (1922).

(62) G. Zemplén, A. Gerecs and Theodora Valatin, *Ber.*, **73B**, 575 (1940).

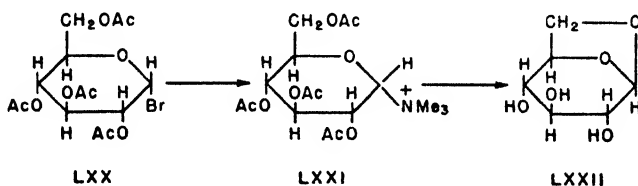
(63) A. E. Knauf, R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **63**, 1447 (1941).

(64) R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **63**, 1484 (1941).

(65) R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **64**, 2435 (1942).

(66) Edna M. Montgomery, N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **64**, 1483 (1942); *ibid.*, **65**, 3, 1848 (1943).

c. *Method of Karrer from 1-Bromo Sugars.* This was proposed as a general method for the preparation of 1,6-anhydro hexoses by Karrer and Smirnof, <sup>69</sup> who applied it in the first instance to the preparation of levoglucosan. Acetobromo-D-glucopyranose (LXX) reacts with trimethylamine to give a quaternary ammonium bromide, the cation of which is represented by LXXI. Treatment of the quaternary salt with



aqueous barium hydroxide eliminates trimethylamine and the acetate groups and yields levoglucosan (LXXII). It is known that acetobromo-D-glucose is an  $\alpha$ -D-sugar (as shown in LXX) and C1 in levoglucosan necessarily has the  $\beta$ -D-configuration (LXXII), but the C1-configuration in LXXI is in doubt. If the inversion occurs during the formation of the quaternary ion, the configuration of LXXI is  $\beta$ -D; if at the stage LXXI  $\rightarrow$  LXXII, then it is  $\alpha$ -D. The opportunity for inversion is present at either stage, since each reaction is an anionic displacement on a carbonium cation.

The method has been successfully used for the preparation of other 1,6-anhydro sugars, e. g., galactosan, <sup>78</sup> although Zemplén and coworkers report a failure to obtain mannosan from acetobromo-D-mannose. <sup>62</sup>

d. *Other Methods.* (1). Certain sugars show a tendency to lose water and form an anhydro sugar in the presence of acids. This is particularly the case with altrose which in acid solution exists as an equilibrium mixture of sugar and anhydride. Richtmyer and Hudson have isolated the altrosan in crystalline form and have shown it to be 1,6-anhydro-D-altropyranose. <sup>70, 71</sup> The anhydro-epiglucosamine of Levene and Meyer <sup>72</sup> is a 3-amino-D-altrose and probably has the 1,6-anhydro ring.

(2). 1,2-Anhydro-D-glucose results from the action of anhydrous ammonia on 1-chloro-2-trichloroacetyl-D-glucose triacetate (Brigl's compound <sup>73, 8</sup>) and the preferential formation of an ethylene oxide ring is undoubtedly due to the lability of the trichloroacetyl group in position 2.

(69) P. Karrer and A. P. Smirnof, *Helv. Chim. Acta*, **4**, 819 (1921).

(70) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **61**, 214 (1939).

(71) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **62**, 961 (1940).

(72) P. A. Levene and G. M. Meyer, *J. Biol. Chem.*, **55**, 221 (1922).

(73) P. Brigl, *Z. physiol. Chem.*, **116**, 1 (1921).



It is of interest that there appears to be no tendency for the establishment of a 1,6-anhydro ring as a result of the saponification of a 1-halogen substituted sugar. In the special case of 1-chloro-2,3,6-trimethyl-D-glucose, however, anhydride formation does occur. The agent for removing the chlorine ion is sodium dust in dry ether.<sup>33, 74</sup> The product can only be 1,4-anhydro-2,3,6-trimethyl-D-glucopyranose and is a representative of that group of dicyclic structures which contain a pyranose and a furanose ring in the same molecule. Another example is the trimethyl-1,4-anhydro-L-idose which was mentioned in Section IV-3. An example from the pentose series is given below.

(3). The unusual formation of an anhydro sugar by the hydrolysis of a trityl derivative has been described.<sup>75</sup> The treatment of 5-trityl-D-ribofuranose triacetate with hydrogen bromide in acetic acid gives a ribosan diacetate which is presumed to be 1,5-anhydro-D-ribofuranose diacetate.

## 2. Constitution and Properties

An outline of the evidence on which the constitution of levoglucosan is based has been given in Section I. Hudson and coworkers have more recently employed the powerful weapon of attack elaborated by them, namely oxidation with per-iodic acid, to establish on the firmest basis the type of ring structures present in the glycosans.<sup>76, 71, 63, 64</sup>

The most stable dicyclic structure produced by any of the methods listed in Section VI-1, is that containing a six-membered (pyranose) and a seven-membered ring together. Where the formation of a seven-membered ring is prevented by substitution at C6, a stable combination of five- and six-membered rings has been observed. Alternatively, 1,2-anhydro sugars are known, but the four-membered anhydro ring has been found in only one glycosan, 1,3-anhydro- $\beta$ -D-galactopyranose, which accompanies the 1,6-anhydro isomer formed by the pyrolysis of  $\alpha$ -D-galactose.<sup>10</sup> One other compound containing a four-membered anhydro ring has been described, namely, 3,5-anhydro-1,2-isopropylidene-D-xylofuranose, which is formed by the saponification of 5-tosyl-1,2-isopropylidene-D-xylofuranose.<sup>9</sup> This anhydro sugar is not, of course, a glycosan. Glycosans are internal glycosides and as such are non-reducing to alkaline agents, do not mutarotate and are hydrolyzed by aqueous acid whereby the parent sugar is regenerated.

(74) K. Freudenberg and E. Braun, *Ber.*, **68B**, 1988 (1935).

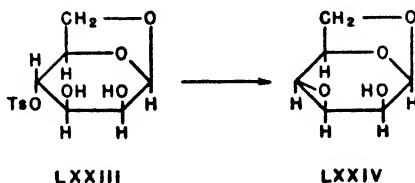
(75) H. Brederick, M. Köthnig and E. Berger, *Ber.*, **73B**, 956 (1940).

(76) E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.*, **62**, 958 (1940).

## VII. THE USE OF ANHYDRO SUGARS IN SYNTHESIS

The ease with which 1,6-anhydro sugars are hydrolyzed by acid and the fact that the original sugar is regenerated thereby makes them of use in the synthesis of various sugar derivatives substituted in positions 2, 3, or 4. 1,6-Anhydro-D-galactose, for example, gives a 3,4-isopropylidene derivative in which only C2 bears a free hydroxyl group. It may therefore be used for the preparation of various 2-substituted derivatives of D-galactose,<sup>78, 79, 86</sup> since both the acetone residue and the 1,6-ring are hydrolyzed by aqueous acid. Similarly, 1,6-anhydro-2,3-isopropylidene-D-mannopyranose is used for the preparation of 4-substituted D-mannoses.<sup>63, 80</sup> This derivative of mannosan has been put to effective use in structurally definitive syntheses of disaccharides by Haskins, Hann and Hudson.<sup>81</sup> On condensing it with acetobromo-D-glucose and acetobromo-D-galactose, the epimers of cellobiose and lactose were respectively obtained. Cellobiose and lactose were in turn prepared from epicellobiose and epilactose by taking advantage of the epimerization method of Bergmann and Schotte<sup>84</sup> in which the intermediates, cellobial and lactal, are prepared.

An anhydro sugar which will probably find extensive synthetic use has been described by Hann and Hudson.<sup>85</sup> This is the tricyclic compound, 3,4:1,6-dianhydro- $\beta$ -D-talopyranose (LXXIV) which is prepared by the saponification of the 4-tosyl derivative of mannosan (LXXIII).



Acetolysis of LXXIV gives, as might be expected, a mixture of the acetates of D-mannose and D-idose and as the dianhydro sugar has a free hydroxyl on C2, it should prove to be a source of 2-substituted D-mannose or D-idose and of 2,4-derivatives of D-mannose and 2,3-derivatives of D-idose.

(78) F. Micheel, *Ber.*, **62B**, 687 (1929).

(79) D. McCreath and F. Smith, *J. Chem. Soc.*, 387 (1939).

(80) W. T. Haskins, R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **65**, 70 (1943).

(81) W. T. Haskins, R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **63**, 1724 (1941); *ibid.*, **64**, 1289, 1490, 1852, (1942).

(84) M. Bergmann and H. Schotte, *Ber.*, **54B**, 440, 1564 (1921).

(85) R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **64**, 925 (1942).

The manifold uses of the ethylene oxide type of anhydro sugar, particularly in regard to the laboratory interconversions of the sugars have already been outlined in Section IV, and the possibilities in this direction are far from exhausted. Scission of the anhydro ring with sodium alkoxides yields alkyl-substituted sugars; with hydrogen chloride, chloro sugars and with ammonia, amino sugars. Derivatives identical with the equivalent derivatives of natural chitosamine were first prepared by Haworth, Lake and Peat<sup>20</sup> from methyl 2,3-anhydro-D-mannoside and the configuration of chitosamine thereby conclusively proved to be that of 2-amino-D-glucose.

The anhydro sugar that has hitherto been most exploited for synthetic purposes is the 5,6-anhydro-1,2-isopropylidene-D-glucufuranose of Ohle and Vargha.<sup>7</sup> Ring cleavage of this compound is effected by a wide variety of acid and alkaline reagents, the chief product being in each case a 6-substituted derivative of D-glucose. Table I summarizes these reactions.

TABLE I  
*Ring Cleavage of 5,6-Anhydro-isopropylidene-D-glucufuranose*

Reagent	Product (substituent for OH on C6 of D-glucose)	References
Hydrogen sulfide, + Ba(OH) <sub>2</sub>	—SH	86
Halogen acid + pyridine + Ac <sub>2</sub> O	—Cl, —Br, —I	86
Carboxylic acid, (RCOOH)	RCOO—	86
Phthalimide	C <sub>6</sub> H <sub>4</sub> (CO) <sub>2</sub> N—	87
Ammonia	—NH <sub>2</sub>	7
Aliphatic amines, RNH <sub>2</sub> , R <sub>2</sub> NH	RNH—, R <sub>2</sub> N—	88
Aromatic primary amines, PhNH <sub>2</sub> , etc.	PhNH—, etc.	89
Diphenylamine, Ph <sub>2</sub> NH	Ph <sub>2</sub> N—	90
Sodium alkoxide, NaOR	—OR	7, 91
$\alpha$ -Amino acid esters, R(NH <sub>2</sub> )·COOEt	—NH·R·COOEt	92
Phenols (PhOH, etc.)	—OPh, etc.	93

(86) H. Ohle and W. Mertens [with, in part, M. Andree and E. Euler], *Ber.*, **68B**, 2176 (1935).

(87) H. Ohle and E. Euler, *Ber.*, **69B**, 1022 (1936).

(88) H. Ohle, E. Euler and W. Malerczyk, *Ber.*, **69B**, 1636 (1936).

(89) H. Ohle, H. Friedeberg and G. Haeseler, *Ber.*, **69B**, 2311 (1936).

(90) H. Ohle and M. Andree, *Ber.*, **71B**, 27 (1938).

(91) H. Ohle and K. Tessmar, *Ber.*, **71B**, 1843 (1938).

(92) B. Helferich and R. Mittag, *Ber.*, **71B**, 1585 (1938).

(93) H. Ohle, E. Euler and R. Voullième, *Ber.*, **71B**, 2250 (1938).

## VIII. TABLES OF PROPERTIES OF ANHYDRO SUGARS AND THEIR DERIVATIVES

TABLE II  
*Anhydro Sugars. Glycosan Type*

Systematic name	Alternative name	M.p., °C.	$[\alpha]_D$	Rotation solvent	References	
1,2-Anhydro- $\alpha$ -D-glucopyranose	$\alpha$ -Glucosan	—	+ 69.6°	H <sub>2</sub> O	61, 61a	
3,4,6-triacetyl-		59.5	+106.5	C <sub>6</sub> H <sub>6</sub>	8, 95	
3,4,6-tribenzoyl-		75			61	
3,4,6-trimethyl-		<25			61	
1,3-Anhydro- $\beta$ -D-galactopyranose		175	+ 54.9	H <sub>2</sub> O	10	
2,4,6-triacetyl-		80	+145	CHCl <sub>3</sub>	10	
1,4-Anhydro-D-glucopyranose						
2,3,6-trimethyl-	{	<25	+ 16.5	H <sub>2</sub> O	33, 74	
1,4-Anhydro-L-idopyranose		<25	- 14.6	CHCl <sub>3</sub>	33, 74	
2,3,6-trimethyl-		<25	+114.2	H <sub>2</sub> O	32	
1,4-Anhydro-?-hexopyranose		<25	+ 90.8	CHCl <sub>3</sub>	32	
2,3,6-trimethyl-		9	- 1.8	H <sub>2</sub> O	34	
			- 0.8	CHCl <sub>3</sub>	34	
1,4-Anhydro-D-ribopyranose		230	+ 78	H <sub>2</sub> O	75	
2,3-diacetyl-		169	-	—	75	
		178	- 66.5	H <sub>2</sub> O	2, 69, 65	
1,6-Anhydro- $\beta$ -D-glucopyranose		Levoglucozan	- 70.5	EtOH	2, 69, 65	
			- 77.5	EtOAc	2, 69, 65	
2,3,4-tribenzoyl-			194	—	—	2, 69
2,3,4-triacetyl-			108	- 45.5	EtOH	2, 69
2,3,4-tribenzyl-			90	- 29.5	CHCl <sub>3</sub>	96
2,4-dibenzyl-			103	- 28.7	CHCl <sub>3</sub>	96
2,4-dibenzyl-3-tosyl-	106		- 5.7	CHCl <sub>3</sub>	96	
2,4-diacetyl-3-tosyl-	87		—	—	96	

(95) J. Hickenbottom, *J. Chem. Soc.*, 3140 (1928); 1676 (1929).(96) G. Zemplén, Z. Csürös and S. Angyal, *Ber.*, 70B, 1848 (1937).

TABLE II (Continued)

Systematic name	Alternative name	M.p., °C.	$[\alpha]_D$	Rotation solvent	References
1,6-Anhydro- $\beta$ -D-galactopyranose	Galactosan	221	- 21.9°	H <sub>2</sub> O	78, 65
2,3,4-triacetyl-		74	- 5.7	CHCl <sub>3</sub>	78, 65
2,3,4-trimethyl-		61	- 69.2	EtOH	79
2,3,4-tribenzoyl-		90	+ 84.8	CHCl <sub>3</sub>	65
2,3,4-tritosyl-		104	- 51.1	CHCl <sub>3</sub>	65
3,4-isopropylidene-		152	- 61.3	H <sub>2</sub> O	78, 79
			- 72.5	CHCl <sub>3</sub>	
2-methyl-		< 25	- 84.5	EtOH	79
2-acetyl-		137	- 51.4	CHCl <sub>3</sub>	65
2-benzoyl-		120	+ 6.3	CHCl <sub>3</sub>	65
2-tosyl-		119	- 63.7	CHCl <sub>3</sub>	65
2-benzoyl-3,4-diacetyl-		104	+ 85.4	CHCl <sub>3</sub>	65
2-benzoyl-3,4-ditosyl-		120	+ 78.0	CHCl <sub>3</sub>	65
2-methyl-		< 25	—	—	79
2-benzoyl-		164	+ 47.2	CHCl <sub>3</sub>	65
2-tosyl-		114	- 20.7	CHCl <sub>3</sub>	97a
1,6-Anhydro- $\beta$ -D-mannopyranose	Mannosan	211	- 127.6	H <sub>2</sub> O	62, 63
2,3,4-triacetyl-		91	- 103.6	H <sub>2</sub> O	62, 63
			- 124.1	CHCl <sub>3</sub>	62, 63
2,3,4-tribenzoyl-		112	- 185.2	CHCl <sub>3</sub>	63
2,3,4-tritosyl-		208	+ 29.7	CHCl <sub>3</sub>	63
2,3,4-trimethyl-		52	- 65.5	H <sub>2</sub> O	62
2,3-isopropylidene-		162	- 58.8	H <sub>2</sub> O	63
4-acetyl-		102	- 72.2	CHCl <sub>3</sub>	63
4-benzoyl-		135	- 103.5	CHCl <sub>3</sub>	63
4-tosyl-		145	- 39.8	CHCl <sub>3</sub>	63, 85
4-methyl-		54	- 33.4	CHCl <sub>3</sub>	63, 80
1,6-Anhydro- $\beta$ -D-altropyranose	Altrosan	Indef.	- 213	H <sub>2</sub> O	70, 71, 97
2,3,4-triacetyl-		101	- 172	CHCl <sub>3</sub>	70, 71, 97
3-amino- (HCl salt)	Anhydro-epi-glucosamine	d. 216	- 172	H <sub>2</sub> O	72
1,6-Anhydro- $\beta$ -D-talopyranose					
3,4-anhydro-	3,4-Anhydro-talosan	74	- 49.5	H <sub>2</sub> O	85
2-tosyl-		148	- 19	CHCl <sub>3</sub>	85

(97) N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, **63**, 1727 (1941).(97a) R. M. Hann and C. S. Hudson, *J. Am. Chem. Soc.*, **68**, 1867 (1946).

TABLE III  
*Anhydro Sugars. Ethylene Oxide Type.*

	M.p., °C.	$[\alpha]_D$	Rotation solvent	Refer- ences
A. <i>2,3-Anhydro hexoses</i>				
Methyl 2,3-anhydro- $\alpha$ -D-manno- pyranoside	—	—	—	—
4,6-benzylidene-	147	+107.4°	CHCl <sub>3</sub>	98, 99
Methyl 2,3-anhydro- $\beta$ -D-manno- pyranoside	<25	- 25*	H <sub>2</sub> O	100
4,6-benzylidene-	183	- 30.7	CHCl <sub>3</sub>	10
4,6-dimethyl-	69	+ 24	H <sub>2</sub> O	100, 19
		+ 40	EtOAc	
Methyl 2,3-anhydro- $\alpha$ -D-allopyranoside	—	—	—	—
4,6-benzylidene-	200	+140.4	CHCl <sub>3</sub>	98, 11, 97
4,6-ethylidene-	128	+100	CHCl <sub>3</sub>	10
4,6-dimethyl-	63	+189	CHCl <sub>3</sub>	101, 24, 11
Methyl 2,3-anhydro- $\beta$ -D-allopyranoside	61	- 6.1	EtOAc	11
4,6-dimethyl-	51	+ 35.3	CHCl <sub>3</sub>	11
4,6-benzylidene-	138	- 15.6	CHCl <sub>3</sub>	11
Methyl 2,3-anhydro- $\beta$ -D-allofuranoside				
5,6-dibenzoyl-	<25	- 96.2	EtOH	15
5,6-ditosyl-	116	- 26.3	CHCl <sub>3</sub>	15
5-tosyl-6-benzoyl-	111	- 45.0	CHCl <sub>3</sub>	15

\* Measured at 5780 Å.

(98) G. J. Robertson and C. F. Griffith, *J. Chem. Soc.*, 1193 (1935).

(99) G. J. Robertson and W. Whitehead, *J. Chem. Soc.*, 319 (1940).

(100) W. N. Haworth, E. L. Hirst and L. Panizzon, *J. Chem. Soc.*, 154 (1934).

(101) D. S. Mathers and G. J. Robertson, *J. Chem. Soc.*, 1076 (1933).

TABLE III (Continued)

	M.p., °C.	$[\alpha]_D$	Rotation solvent	Refer- ences
<b>B. 3,4-Anhydro hexoses</b>				
Methyl 3,4-anhydro- $\beta$ -D-galacto- pyranoside	158	-118°	H <sub>2</sub> O	102, 12
2,6-diacetyl-	118	-114	CHCl <sub>3</sub>	102
2,6-dibenzoyl-	133	- 80	CHCl <sub>3</sub>	102
2,6-dimethyl-	84	-148.2	CHCl <sub>3</sub>	23
2-acetyl-6-trityl-	181	- 91.8	CHCl <sub>3</sub>	23
6(?) -methyl-	121	-141.6	H <sub>2</sub> O	23
Phenyl 3,4-anhydro- $\beta$ -D-galacto- pyranoside	150	-161	H <sub>2</sub> O	103
2,6-diacetyl-	123	-104.5	CHCl <sub>3</sub>	103
Methyl 3,4-anhydro- $\beta$ -D-allopyranoside	—	—	—	—
2,6-dimethyl-	46	-144.5	CHCl <sub>3</sub>	11, 104
Methyl 3,4-anhydro- $\beta$ -D-altro- pyranoside	—	—	—	—
2,6-dimethyl-	<25	- 21.0	H <sub>2</sub> O	27
3,4-Anhydro- $\beta$ -D-tagatopyranose	145	- 56.0	H <sub>2</sub> O	25
		→		
1,2-isopropylidene-	82	+ 16.8	H <sub>2</sub> O	25
		- 60.0	CHCl <sub>3</sub>	
5-acetyl-	81	- 80.7	CHCl <sub>3</sub>	25
5-tosyl-	118	- 28.6	CHCl <sub>3</sub>	25
5- $\beta$ -naphthosulfonyl-	d. 140	- 27.0	CHCl <sub>3</sub>	25
3,4-Anhydro- $\beta$ -D-psicopyranose	—	- 38.7	CHCl <sub>3</sub>	—
1,2-isopropylidene-	92	—	—	—
5-acetyl-	81	- 47.6	CHCl <sub>3</sub>	18
5-benzoyl-	112	+ 5.9	CHCl <sub>3</sub>	18
5-tosyl-	95	+ 24.0	CHCl <sub>3</sub>	18
5-methyl-	<25	+ 4.7	CHCl <sub>3</sub>	18
		- 2.8	CHCl <sub>3</sub>	18
<b>C. 5,6-Anhydro hexoses</b>				
5,6-Anhydro-D-glucofuranose				
1,2-isopropylidene-	133	- 26.5	H <sub>2</sub> O	7, 6

(102) B. Helferich and A. Müller, *Ber.*, **63B**, 2142 (1930).(103) B. Helferich and F. Strauss, *J. prakt. Chem.*, **142**, 13 (1935).(104) K. Freudenberg and E. Plankenhorn, *Ann.*, **536**, 257 (1938).

TABLE IV  
*Anhydro Sugars. Propylene Oxide Type*

	M.p., °C.	$[\alpha]_D$	Rotation solvent	Refer- ence
3,5-Anhydro-D-xylofuranose 1,2-isopropylidene-	{ 17	+14.7° +11.7	H <sub>2</sub> O CHCl <sub>3</sub>	9

TABLE V  
*Anhydro Sugars. Hydrofuranol Type  
2,5-Anhydrides*

Systematic name	Alternative name	M.p., °C.	$[\alpha]_D$	Rotation solvent	Refer- ences
2,5-Anhydro-D-mannose tribenzoyl- methyl glycoside · H <sub>2</sub> O	Chitose	amorph. 116 <sup>7</sup> 169	+33.2° ± 0 —	H <sub>2</sub> O — —	4, 105, 106 105 105
2,5-Anhydro-D-glucose	Epichitose	d. 240	— 96	H <sub>2</sub> O	106
2,5-Anhydro-D-talose (?)	Chondrose	< 25	+ 20	H <sub>2</sub> O	106
2,5-Anhydro-D-mannonic acid	Chitonic acid	{ < 25	+44.5 +38.3	H <sub>2</sub> O 5% HCl	4 107
Ca salt · 2H <sub>2</sub> O		—	+30 → +35	H <sub>2</sub> O	4, 53, 108 56, 107
brucine salt		222	— 8.5		108
2,5-Anhydro-D-gluconic acid	Chitaric acid	—	+70.3	H <sub>2</sub> O	107
Ca salt · 4H <sub>2</sub> O		—	+63; +70	H <sub>2</sub> O	53, 108, 107
brucine salt		195	— 3.0	H <sub>2</sub> O	108, 107
2,5-Anhydro-D-talonic acid					
Brucine salt · H <sub>2</sub> O		218	— 12.4	H <sub>2</sub> O	109
2,5-Anhydro-D-galactonic acid					
brucine salt		244	— 9.4		109

(105) C. Neuberg (with H. Wolff and W. Neimann), *Ber.*, **35**, 4009 (1902).

(106) P. A. Levene and R. Ulpts, *J. Biol. Chem.*, **64**, 475 (1925).

(107) P. A. Levene, *J. Biol. Chem.*, **59**, 135 (1924).

(108) P. A. Levene and G. M. Meyer, *J. Biol. Chem.*, **26**, 364 (1916).

(109) P. A. Levene, *J. Biol. Chem.*, **31**, 609 (1917).



TABLE V (Continued)

Systematic name	Alternative name	M.p., °C.	$[\alpha]_D$	Rotation solvent	References
2,5-Anhydro-D-manno-saccharic acid	Isosaccharic acid	185	+46.1°	H <sub>2</sub> O	51, 52, 4, 110
Ca salt		-	+28	H <sub>2</sub> O	110
diethyl ester		101	+35.5	H <sub>2</sub> O	
diacetate		49	—		51, 52, 4
diamide		226	+ 7.25	H <sub>2</sub> O	51, 52, 4
dianilide		231	—	—	51, 52, 4
2,5-Anhydro-D-glucosaccharic acid	Epi-isosaccharic acid	160	+39.7	H <sub>2</sub> O	111, 55, 110
KH salt · $\frac{1}{2}$ H <sub>2</sub> O		—	+38.5	H <sub>2</sub> O	110
Ca salt		—	+28	H <sub>2</sub> O	110
2,5-Anhydro-L-glucosaccharic acid		163	-38.8	H <sub>2</sub> O	55, 56
KH salt · H <sub>2</sub> O		—	-38	H <sub>2</sub> O	55, 56
2,5-Anhydro-D-allosaccharic acid	Chondrosic acid, Anhydrotalomic acid	180	-16.6	H <sub>2</sub> O	111, 109, 56
Ca salt · H <sub>2</sub> O		—	- 9.0	H <sub>2</sub> O	111, 109
2,5-Anhydro-galactosaccharic acid	Epi-chondrosic acid, Anhydromucic acid	205	inactive	H <sub>2</sub> O	111, 109
2,5-Anhydro-allosaccharic acid					
Ca salt		—	inactive	H <sub>2</sub> O	112, 49
2,5-Anhydro-D-idosaccharic acid · 2H <sub>2</sub> O		—	-93.4	H <sub>2</sub> O	55, 56
		226	-78.0	H <sub>2</sub> O	56

(110) P. A. Levene, *J. Biol. Chem.*, **39**, 69 (1919).

(111) P. A. Levene and F. B. LaForge, *J. Biol. Chem.*, **20**, 433 (1915).

(112) P. A. Levene and E. P. Clarke, *J. Biol. Chem.*, **46**, 19 (1921).

(113) H. Ohle and E. Euler, *Ber.*, **63B**, 1796 (1930).

TABLE VI  
*Anhydro Sugars. Hydrofuranol Type*  
*3,6-Anhydrides*

	M.p., °C.	$[\alpha]_D$	Rotation solvent	References
3,6-Anhydro-D-glucose	118	+ 53.8°	H <sub>2</sub> O	3, 5, 44, 28, 15
5-acetyl-	< 25	+ 91.2	CHCl <sub>3</sub>	113
1,2,5-triacetyl-	< 25	+ 178.9	CHCl <sub>3</sub>	113
5-tosyl- ( $\alpha$ -form)	100	+ 64.8	CHCl <sub>3</sub>	113
		+ 56.5	Me <sub>2</sub> CO	113
		+ 66.7 $\rightarrow$ + 57.9	Dry EtOH	113
5-tosyl-1-chloro-2-acetyl-	105	+ 194	CHCl <sub>3</sub>	113
5-tosyl-1-bromo-2-acetyl-	89	+ 220.2	CHCl <sub>3</sub>	113
4-methyl-	< 25	- 17	H <sub>2</sub> O	28
2,4-dimethyl-	< 25	- 28	H <sub>2</sub> O	28
2,5-dimethyl-	< 25	+ 110 $\rightarrow$ + 120	H <sub>2</sub> O	28, 11
anilide of	96	+ 143	EtOH	28, 11
2,4,5-trimethyl	< 25	--	--	28
dimethyl acetal of	< 25	- 6	H <sub>2</sub> O	28
1,2-isopropylidene-	56-57	+ 29.3	H <sub>2</sub> O	5
5-benzoyl-	58-59	+ 22.3	CHCl <sub>3</sub>	5
5-acetyl-	31	+ 74.8	CHCl <sub>3</sub>	113
5-tosyl-	132	+ 39.3	CHCl <sub>3</sub>	5
5-methyl- (+ 1H <sub>2</sub> O)	43-44	+ 82	EtOH	28
Methyl 3,6-anhydro- $\alpha$ -D-glucoside	70	+ 164	H <sub>2</sub> O	28
2,5-dimethyl-	45	+ 208	H <sub>2</sub> O	28
Methyl 3,6-anhydro- $\beta$ -D-glucoside	98	- 54	H <sub>2</sub> O	15, 28
2,5-dimethyl-	< 25	+ 15	H <sub>2</sub> O	28
2,5-dibenzoyl-	99	+ 2.8	CHCl <sub>3</sub>	15
2,5-ditosyl-	130.5	+ 55.7	CHCl <sub>3</sub>	15
Methyl 3,6-anhydro- $\alpha$ -D-glucopyranoside	{ 89-95	+ 40	H <sub>2</sub> O	114
	108	+ 56	H <sub>2</sub> O	28
4-methyl-	152	+ 24	H <sub>2</sub> O	28
2,4-dimethyl-	66	+ 50	H <sub>2</sub> O	28

(114) B. Helferich, W. Klein and W. Schäfer, *Ber.*, 59B, 79 (1926).

TABLE VI (Continued)

	M.p., °C.	$[\alpha]_D$	Rotation solvent	References
Methyl 3,6-anhydro- $\beta$ -D-glucopyranoside	<25	-138°	H <sub>2</sub> O	3, 28
2,4-dimethyl-	<25	- 96	H <sub>2</sub> O	28
2,4-diacetyl-	78-79	-107	CHCl <sub>3</sub>	104
3,6-Anhydro-D-gluconic acid	123-125	—	—	35
$\gamma$ -lactone	115	+82.3→+66.4	H <sub>2</sub> O	35
5-methyl-	<25	+109→+71	H <sub>2</sub> O	28
2,5-dimethyl-	<25	+96→+73	H <sub>2</sub> O	28
	<25	+91→+64	H <sub>2</sub> O	11
	149	+ 77.8	H <sub>2</sub> O	35
amide	160	+109	H <sub>2</sub> O	28
5-methyl-	136-137	+ 68	H <sub>2</sub> O	28
2,5-dimethyl-	92	+ 41	H <sub>2</sub> O	28, 11
4-methyl-	—	- 7.5	H <sub>2</sub> O	28
2,4-dimethyl-	155	+ 63	H <sub>2</sub> O	28
4-methyl-	—	—	—	—
methyl ester of	<25	+ 2	H <sub>2</sub> O	28
2,4-dimethyl-	156	+ 52	H <sub>2</sub> O	28
3,6-Anhydro-D-galactose	—	+ 27.5	H <sub>2</sub> O	13
	—	+ 24	H <sub>2</sub> O	38
dimethyl acetal of	<25	+ 36.5	H <sub>2</sub> O	38
2,4,5-tribenzoyl-	112	—	—	38
2,4-dimethyl-	<25	+ 24	H <sub>2</sub> O	38
anilide of	122	—	—	38, 115
dimethyl acetal of	36	+ 36	H <sub>2</sub> O	38
2,4,5-trimethyl-	<25	+ 41	H <sub>2</sub> O	38
dimethyl acetal of	<25	+ 41	H <sub>2</sub> O	38
Methyl 3,6-anhydro- $\alpha$ -D-galactopyranoside	140	+ 84	H <sub>2</sub> O	116, 13, 38, 42
2-methyl-	102	+ 88	H <sub>2</sub> O	40
4-methyl-	55	+ 75	H <sub>2</sub> O	40
2,4-dimethyl-	<25	+ 73	H <sub>2</sub> O	38
2-tosyl-	138	+ 56	CHCl <sub>3</sub>	40
2-tosyl-4-methyl-	126	+ 88	CHCl <sub>3</sub>	40
Methyl 3,6-anhydro- $\beta$ -D-galactopyranoside	119	-115	H <sub>2</sub> O	117, 115, 118, 38, 42
2,4-dimethyl-	83	- 77	H <sub>2</sub> O	38

(115) E. G. V. Percival, J. C. Somerville and I. A. Forbes, *Nature*, **142**, 797 (1938);  
 I. A. Forbes and E. G. V. Percival, *J. Chem. Soc.*, 1844 (1939).

(116) F. Valentin, *Collection Czechoslov. Chem. Commun.*, **4**, 364 (1932).

(117) W. N. Haworth, J. Jackson and F. Smith, *Nature*, **142**, 1075 (1938).

(118) E. G. V. Percival and I. A. Forbes, *Nature*, **142**, 1076 (1938).

TABLE VI (Continued)

	M.p., °C.	$[\alpha]_D$	Rotation solvent	References
3,6-Anhydro-D-galactonic acid	<25	+ 33°	H <sub>2</sub> O	38
methyl ester	<25	+ 38	H <sub>2</sub> O	38
2,4-dimethyl-	152	+ 66	H <sub>2</sub> O	38
methyl ester	51	+ 67	H <sub>2</sub> O	38
amide	151	+ 81	H <sub>2</sub> O	38
2,4,5-trimethyl-	<25	+ 64	H <sub>2</sub> O	38
methyl ester	<25	+ 67	H <sub>2</sub> O	38
brucine salt	114	- 3	H <sub>2</sub> O	38
3,6-Anhydro-L-galactose				
2,4-dimethyl-	114	- 23	H <sub>2</sub> O	119, 115
anilide	117	—	—	115
Methyl 3,6-anhydro-β-L-galacto- pyranoside				
2,4-dimethyl-	{ 83	+ 85.3	CHCl <sub>3</sub>	119, 115
		+ 75	H <sub>2</sub> O	119, 115
3,6-Anhydro-L-galactonic acid	—	—	—	—
2,4-dimethyl-	—	—	—	—
methyl ester	{ 49	- 72.5	CHCl <sub>3</sub>	115
		- 64	H <sub>2</sub> O	
amide	151	- 74	H <sub>2</sub> O	115
2,5-dimethyl-	160	- 65	H <sub>2</sub> O	120
amide	173	—	—	121, 120
3,6-Anhydro-D-mannose	103	+ 95.9	H <sub>2</sub> O	14
benzylphenylhydrazone	145	+ 43.6	MeOH	14
Methyl 3,6-anhydro-α-D-manno- furanoside	85	+ 157	H <sub>2</sub> O	14
Methyl 3,6-anhydro-α-D-manno- pyranoside	131	+ 97.1	H <sub>2</sub> O	14, 42
3,6-Anhydro-D-mannonic acid				
γ-lactone	113	+ 126.5 → + 115.3	H <sub>2</sub> O	122
phenylhydrazide	d. 190	+ 19.7	MeOH	122
3,6-Anhydro-D-allose				
phenylosazone	165-168	- 138	C <sub>6</sub> H <sub>5</sub> N- MeOH(1:1)	44, 45
2-methyl-	{ <25	+ 106.3	H <sub>2</sub> O	
		+ 81.3	CHCl <sub>3</sub>	99
Methyl 3,6-anhydro-α-D-altro- pyranoside	—	—	—	—
2-methyl-	108	+ 105.1		99
2,4-dimethyl-	liq.	+ 69.0	CHCl <sub>3</sub>	99
3,6-Anhydro-L-idose	106	+ 25.4	H <sub>2</sub> O	116
1,2-isopropylidene-	105	+ 24.9	H <sub>2</sub> O	116

(119) S. Hands and S. Peat, *Nature*, **142**, 797 (1938).(120) E. G. V. Percival and T. G. H. Thomson, *J. Chem. Soc.*, 750 (1942).(121) W. G. M. Jones and S. Peat, *J. Chem. Soc.*, 225 (1942).(122) F. Valentin, *Collection Czechoslov. Chem. Commun.*, **9**, 315 (1937).



# ANALOGS OF ASCORBIC ACID

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## I. SYNTHESIS OF ANALOGS OF ASCORBIC ACID

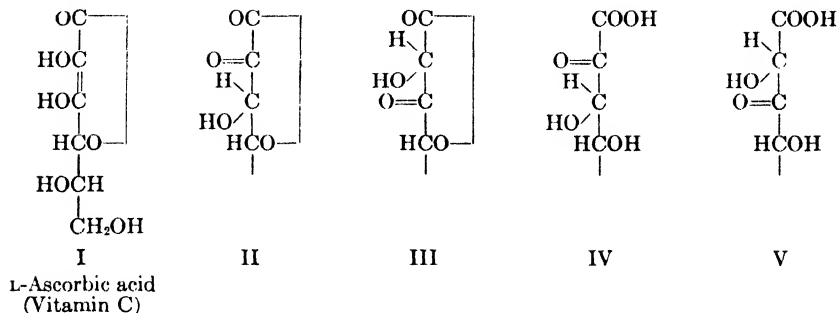
Vitamin C or L-ascorbic acid, the antiscorbutic vitamin, has been shown by degradative<sup>1</sup> and synthetic methods<sup>2,3</sup> to have the structure I.

(1) R. W. Herbert, E. L. Hirst, E. G. V. Percival, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.*, 1270 (1933).

(2) T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, 16, 561, 1019 (1933); 17, 510 (1934).

(3) R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith and M. Stacey, *J. Chem. Soc.*, 1419 (1933); D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *J. Chem. Soc.*, 62 (1934).

The unique feature of L-ascorbic acid and its analogs<sup>4</sup> lies in the enediolic system and it is this system which is responsible for the remarkable reducing properties displayed by these substances.

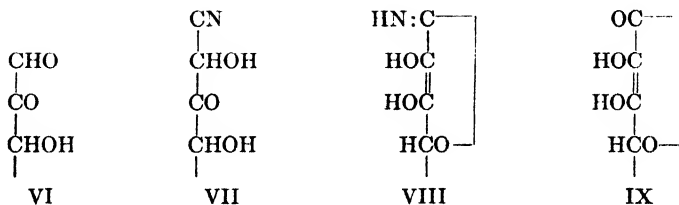


It will be seen that the enediolic system can theoretically be written in the isomeric 2-keto (II) or 3-keto (III) forms and these in turn are seen to be derived from the 2-keto and the 3-keto acids IV and V, respectively (compare with benzoin which reacts with iodine in an analogous fashion to L-ascorbic acid). Consequently the synthesis of L-ascorbic acid and of its analogs has consisted in devising methods for the formation of 2-keto or 3-keto hydroxy acids followed by their enolization and lactonization. Four main methods are available for the synthesis of analogs of L-ascorbic acid containing the characteristic five-membered unsaturated enediolic ring.

1. Addition of hydrogen cyanide to osones followed by hydrolysis.
2. Simultaneous lactonization and isomerization of 2-keto acids or esters.
3. Condensation of hydroxy aldehydes with ethyl glyoxylate or ethyl mesoxalate.
4. Condensation of esters of hydroxy acids.

#### 1. Addition of Hydrogen Cyanide to Osones Followed by Hydrolysis

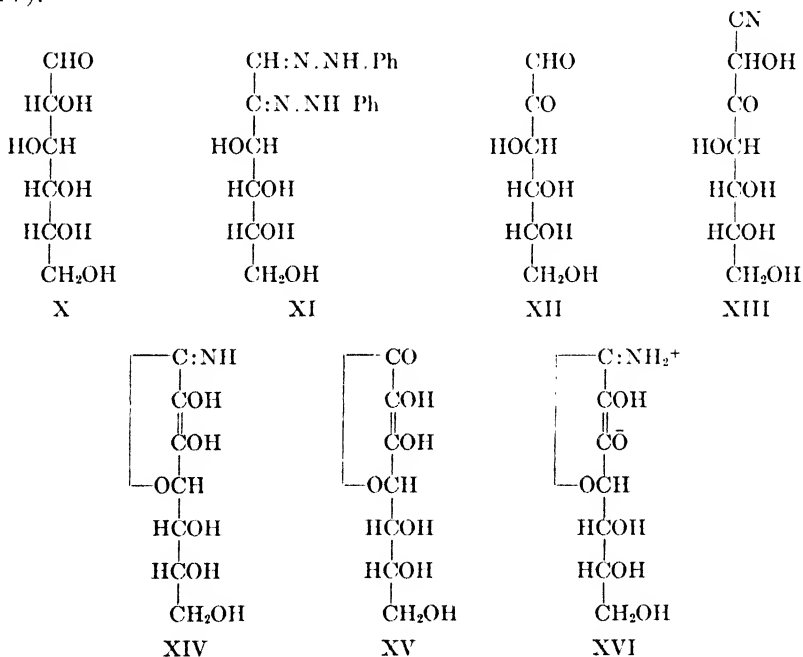
In this reaction the osone (or  $\alpha$ -keto aldehyde (VI)) is allowed to react with hydrogen cyanide or preferably with potassium cyanide in aqueous



(4) T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **17**, 311 (1934).

solution.<sup>2,3</sup> Condensation occurs with the aldehydic group as in the usual cyanohydrin formation and in the Kiliani synthesis of hydroxy nitriles of the sugar series. The nitrile (VII) is not isolated; instead, ring formation takes place to give an imino group at C1 accompanied by enolization, and there is formed an imino analog of ascorbic acid (VIII). The formation of this stable imino structure is facilitated in an alkaline medium, as a result of which potassium cyanide can be utilized instead of hydrogen cyanide. Hydrolysis of the "imino-ascorbic acid" eliminates the imino group which is replaced by a carbonyl group giving the ascorbic acid ring system IX.

This method, utilized simultaneously by Reichstein<sup>2</sup> and by Haworth and coworkers<sup>3</sup> in the first synthesis of D- or L-ascorbic acid, can be illustrated in detail by reference to the preparation of D-glucoscorbic acid (XV).<sup>5</sup>



D-Glucosone (XII), prepared from D-glucose phenyllosazone (XI) by the action of concentrated hydrochloric acid, is treated in aqueous solution with potassium cyanide. The "imino-D-glucoscorbic" acid (XIV) which readily separates shows many of the properties of ascorbic acid. Thus it shows a strong selective absorption band in the ultra-

(5) W. N. Haworth, E. L. Hirst, J. K. N. Jones and F. Smith, *J. Chem. Soc.*, 1192 (1934).



violet region of the spectrum, it reduces silver nitrate, Fehling's solution, and decolorizes potassium permanganate immediately. As in the case of L-ascorbic acid, the imino compound reacts with iodine in aqueous solution, thus affording a method for its estimation. Evidence from an examination of the optical rotatory dispersion of XIV shows that it resembles sodium L-ascorbate which is formed by loss of the more active hydrogen ion from C3. This observation suggests that in aqueous solution the imino-D-glucoscorbic acid possesses the internal salt-like structure XVI. It would appear that in the presence of mineral acid (excess hydrogen ions) separation of the proton from the C3-OH group is suppressed, a conjecture supported by the fact that the optical rotatory dispersion of a solution of imino-D-glucoscorbic acid in the presence of mineral acid is similar to that of L-ascorbic acid.

When the imino compound XIV is heated with dilute hydrochloric acid, hydrolysis of the imino group takes place and there is formed ammonium chloride and D-glucoscorbic acid (XV). The latter displays all the chemical properties of L-ascorbic acid.

A similar series of experiments<sup>6</sup> has also been carried out starting with D-galactosone prepared from D-galactose phenylosazone. The intermediate imino-D-galactoscorbic acid was obtained crystalline and this in turn afforded on hydrolysis the corresponding crystalline D-galactoscorbic acid. There is little doubt therefore that an imino compound analogous to XIV is an intermediate product in all the syntheses of L-ascorbic acid and its analogs by this method.

The above method has proved to be of general application and of great value in the synthesis of analogs of L-ascorbic acid. In order to facilitate the isolation of the ascorbic acids it is important to obtain the osones in as pure a state as possible and this depends to a large extent upon the initial isolation of a pure osazone. Experiments have demonstrated that if the osazone is soluble in ethyl alcohol (as is usually the case with those osazones prepared from pentose sugars) it is advisable to convert the osazone into the osone by the agency of benzaldehyde.<sup>6</sup> On the other hand, if the osazone is sparingly soluble in alcohol, the osone is best prepared by decomposition of the osazone with concentrated hydrochloric acid.<sup>7</sup> It has been reported that osones can be obtained by the direct oxidation of sugars with cupric salts.<sup>8</sup>

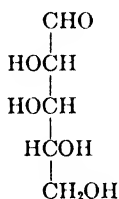
The nomenclature adopted in the designation of the analogs of L-ascorbic acid is based for convenience upon the name of the sugar from which the osone was prepared. For example, D-xylose (XVIII)

(6) E. Fischer and E. F. Armstrong, *Ber.*, **35**, 3141 (1902).

(7) E. Fischer, *Ber.*, **22**, 87 (1889).

(8) I. Stone, U. S. Pat. 2,206,374 (1940).

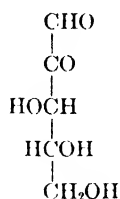
gives D-xylosone (XIX), and the latter by the method given above yields D-xyloascorbic acid or simply D-ascorbic acid (XX), the enantiomorph of the naturally occurring L-ascorbic acid or Vitamin C. It should be borne in mind, however, that it might equally well be called D-lyxoascorbic acid since D-lyxose (XVII) and D-xylose (XVIII), which afford the same osone, will also give the same osone.



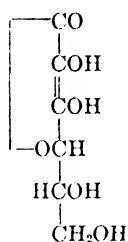
XVII



XVIII



XIX



XX

D-Xyloascorbic acid  
(D-Ascorbic acid)

In addition to L-ascorbic acid this method, involving the use of osones and potassium cyanide, has afforded the following analogs:

- D-Xyloascorbic acid (D-Ascorbic acid) (XX)<sup>2,3</sup>;
- L-Araboascorbic acid (LXXII)<sup>2,3</sup>;
- L-Fucoascorbic acid (LXXI)<sup>8a</sup>;
- L-Rhamnoascorbic acid (LXX)<sup>8b</sup>;
- D-Glucoascorbic acid (XV)<sup>2,3,5</sup>;
- L-Glucoascorbic acid (XLVIII)<sup>8c</sup>;
- D-Galactoascorbic acid (LXXIII)<sup>2,3,5</sup>;
- L-Guloascorbic acid (LXXIV)<sup>2</sup>;
- L-Alloascorbic acid (LXXV)<sup>8d</sup>;
- D-Glucoheptoascorbic acid (XLIV)<sup>29</sup>.

## 2. Simultaneous Lactonization and Enolization of 2-Keto-3,4-dihydroxy Acids or Esters

This method, which utilizes 2-keto-3,4-dihydroxy acids or esters, is by far the best method for the preparation of ascorbic acids but it is dependent upon a successful process for the production of the 2-keto

(8a) T. Reichstein and V. Demole, *Festschrift für E. C. Barell, Basel*, 107 (1936).

(8b) T. Reichstein, L. Schwarz and A. Grüssner, *Helv. Chim. Acta.*, **18**, 353 (1935).

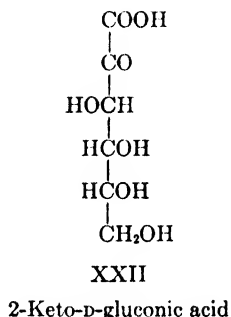
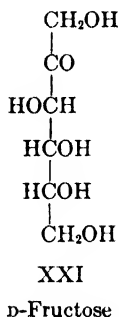
(8c) W. N. Haworth, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 552 (1937)

(8d) M. Steiger, *Helv. Chim. Acta.*, **18**, 1252 (1935).

acids. Once the latter have been obtained their conversion to ascorbic acids can be smoothly effected. Up to the present this method is the most successful for the preparation<sup>4</sup> of synthetic Vitamin C on a commercial scale.

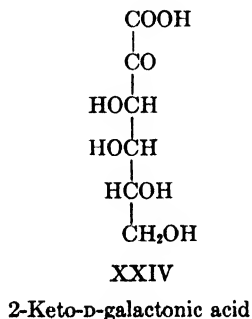
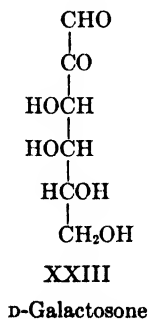
*a. Preparation of 2-Keto Acids*

(1) *Direct Oxidation of Ketose Sugars.* The primary alcoholic group at C1 adjacent to the keto group at C2 of a ketose sugar such as D-fructose (XXI) or L-sorbose, is more sensitive to oxidation than the other primary alcoholic group at C6 or secondary alcoholic groups at C3, C4, and C5.



By careful regulation of conditions, the ketose sugars D-fructose and L-sorbose can be transformed by the agency of nitric acid into the corresponding 2-keto-D-gluconic acid (XXII) and 2-keto-L-gulonic acid (XXVI),<sup>9</sup> respectively.

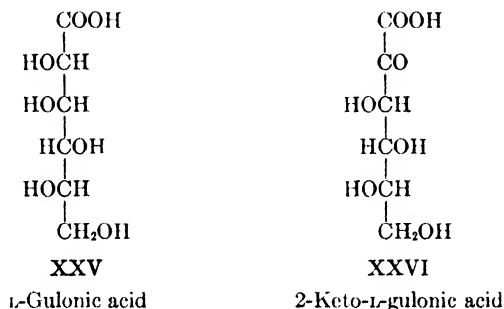
(2) *Oxidation of Osones.*



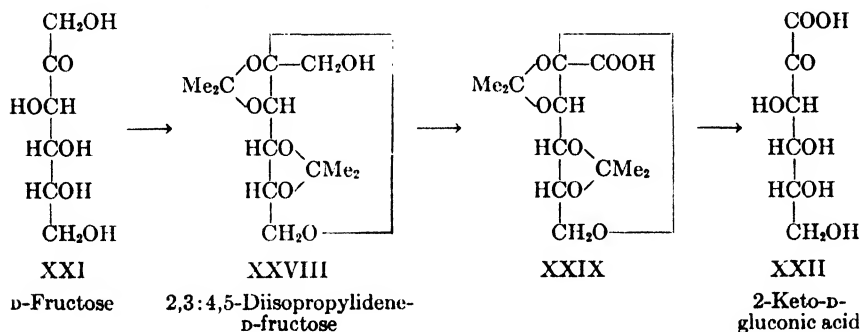
(9) W. N. Haworth, E. L. Hirst, J. K. N. Jones and F. Smith, British Pat. 443,901 (1936); French Pat. 794,221 (1936).

The 2-keto acids such as 2-keto-D-galactonic acid (XXIV) can be derived from the corresponding osone (XXIII) by oxidation with bromine.<sup>10</sup> Oxidation of L-gulosone by the same method has provided 2-keto-L-gulonic acid. The success of this oxidation depends to a large extent upon the purity of the osone subjected to oxidation and this, as previously stated, is controlled by the purity of the osazone.

(3) *Oxidation of Aldonic Acids.* Preferential oxidation of the secondary alcoholic group adjacent to the carboxyl group in sugar acids or aldonic acids such as L-gulonic acid (XXV) can be carried out with chromic acid<sup>12</sup> or with chlorates in the presence of a vanadium catalyst.<sup>13</sup>



(4) *Oxidation of Isopropylidene Derivatives of Ketose Sugars.* Whenever isopropylidene derivatives can be obtained in which all groups except the primary alcoholic grouping at C1 are blocked this method is undoubtedly the best. It may be illustrated by reference to the preparation of 2-keto-D-gluconic acid from D-fructose.<sup>14</sup>



(10) C. Neuberg and T. Kitasato, *Biochem. Z.*, **183**, 485 (1927).

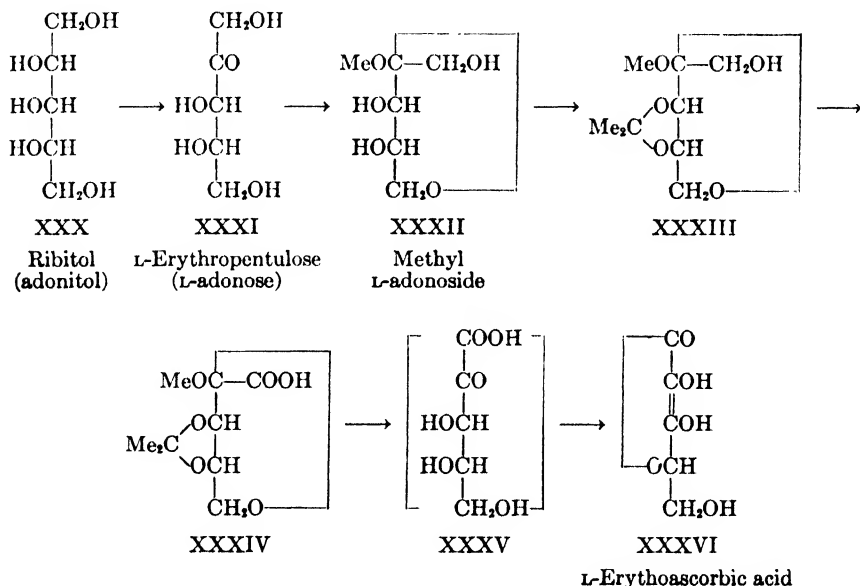
(12) R. Pasternack and P. P. Regna, U. S. Pat. 2,153,311 (1939).

(13) R. Pasternack and P. P. Regna, U. S. Pat. 2,188,777 (1940), P. P. Regna and B. P. Caldwell, *J. Am. Chem. Soc.*, **66**, 243 (1944).

(14) H. Ohle and R. Walter, *Ber.*, **63B**, 843 (1930).

Condensation of D-fructose (XXI) with acetone in the presence of sulfuric acid gives rise to 2,3:4,5-diisopropylidene-D-fructose (XXVIII) which on treatment with potassium permanganate in alkaline solution undergoes oxidation to give 2,3:4,5-diisopropylidene-2-keto-D-gluconic acid (XXIX). Hydrolysis of the latter proceeds smoothly either by boiling an aqueous solution of it or by heating it with dilute sulfuric acid and there results 2-keto-D-gluconic acid (XXII).

A similar series of reactions applied to L-sorbose (once a rare sugar but now easily obtained from sorbitol by bacterial oxidation<sup>15</sup>) gives rise to 2-keto-L-gulonic acid.<sup>4</sup> The method is not confined to keto-hexoses and has been employed in the preparation from L-erythropentulose (XXXI) of the 2-keto-L-ribonic acid (XXXV) which undergoes immediate transformation to the corresponding L-erythroascorbic acid (XXXVI).<sup>16</sup>



Bacterial oxidation of ribitol (adonitol; XXX) affords L-adonose or L-erythropentulose (XXXI) (cf. the bacterial oxidation of sorbitol to L-sorbose). By the agency of methyl alcoholic hydrogen chloride, XXXI yields methyl L-adonoside (XXXII) and condensation of the

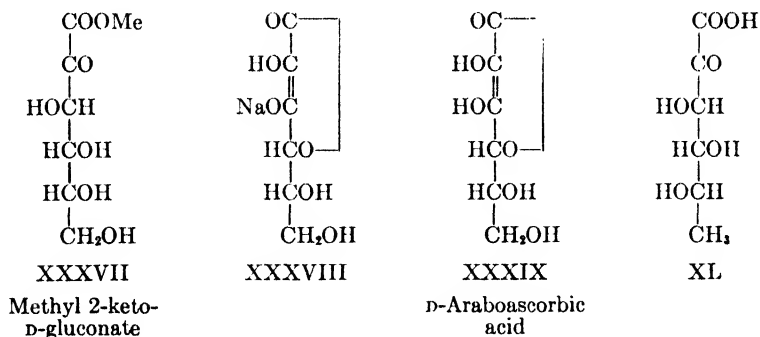
(15) H. H. Schlubach and J. Vorwerk, *Ber.*, **66B**, 1251 (1933); G. Bertrand, *Bull. soc. chim.*, [3] **15**, 627 (1896).

(16) T. Reichstein, *Helv. Chim. Acta*, **17**, 996, 1003 (1934).

latter with acetone gives rise to methyl 3,4-isopropylidene-L-adonoside (XXXIII). This compound, in which only the  $\text{—CH}_2\text{OH}$  group at C1 is available for oxidation, gives upon treatment with potassium permanganate the corresponding acid XXXIV. Elimination of the glycosidic methyl group and the acetone residue by hydrolysis does not give the keto acid XXXV but instead lactonization and enolization takes place simultaneously with the hydrolysis and there is isolated the more stable L-erythroascorbic acid (XXXVI).

*b. Simultaneous Lactonization and Enolization of 2-Keto-3,4-dihydroxy Acids or Esters*

This double transformation was effected by Maurer and Schiedt<sup>17</sup> and also by Ohle, Erlbach and Carls<sup>18</sup> who treated methyl 2-keto-D-gluconate (XXXVII) with sodium methoxide and obtained a product which contained no ester grouping. The product thus isolated was D-araboascorbic acid (XXXIX) but it was not recognized as such by Maurer and Schiedt<sup>17</sup> until Haworth and coworkers<sup>19</sup> pointed out that the product isolated from the reaction was the enantiomorph of L-araboascorbic acid (LXXII) prepared by the osone hydrogen cyanide method. The keto acids of the hexose series can be transformed into the corresponding ascorbic acid derivatives simply by being heated in aqueous



solution. An equilibrium is set up between the keto acid and the corresponding ascorbic acid but since this equilibrium does not lie sufficiently on the ascorbic acid side [compare with 2-keto-L-ribonic acid (XXXV) and 6-desoxy-2-keto-L-gulonic acid (XL)] it has been found preferable to

(17) K. Maurer and B. Schiedt, *Ber.*, **66B**, 1054 (1933); **67B**, 1239 (1934).

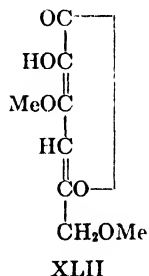
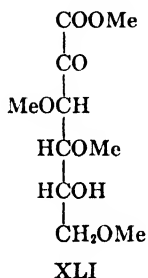
(18) H. Ohle, H. Erlbach and H. Carls, *Ber.*, **67B**, 324 (1934).

(19) D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *J. Chem. Soc.*, 63 (1934).

utilize the esters instead of the free acids. These esters, which can be made either by boiling the keto acids with methyl alcohol containing hydrogen chloride,<sup>4</sup> by titration of the acids with diazomethane,<sup>4</sup> or by boiling the acids in methyl alcohol alone,<sup>9</sup> are almost quantitatively transformed into the corresponding ascorbic acids by means of methyl alcoholic sodium methoxide. The ascorbic-acid-like products separate almost immediately from the hot reaction mixture as the solid sodium salts, thus ensuring the complete conversion of the 2-keto esters into the ascorbic acids. Thus, methyl 2-keto-D-gluconate (XXXVII) gives sodium D-araboascorbate (XXXVIII) and this upon addition of hydrochloric acid yields D-araboascorbic acid (XXXIX).<sup>17,18,20</sup> By a similar procedure methyl 2-keto-L-gulonate can be converted by means of sodium methoxide into L-ascorbic acid.<sup>4</sup> Much saving of time and material has been effected by the discovery that the diisopropylidene-2-keto acids can be directly transformed into the ascorbic acids by heating them in a solution containing hydrogen chloride. The solution is best made in a mixture of solvents such as chloroform and ethyl alcohol of such proportions that it will dissolve the diisopropylidene-keto acid and hydrogen chloride and yet will not dissolve the ascorbic acid which is formed. By this means ascorbic acid separates from the reaction mixture in the crystalline form.<sup>21</sup>

These 2-keto esters can also be transformed into the corresponding ascorbic acids by heating them in aqueous solution with magnesium, iron, nickel, cobalt, manganese, cadmium and zinc.<sup>20a</sup>

The process of lactonization and enolization of 2-keto esters under the influence of alkaline reagents has also been applied to the production of analogs of L-ascorbic acid containing a six-membered ring structure.<sup>22</sup> For example, methyl 3,4,6-trimethyl-2-keto-D-gluconate (XLI) is treated



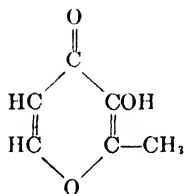
(20) Doreen Heslop, Elizabeth Salt and F. Smith, *J. Chem. Soc.*, 225 (1944).

(20a) R. Pasternack and P. P. Regna, *U. S. Pats.* 2,165,151; 2,165,184 (1939).

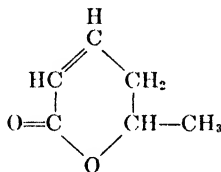
(21) T. Reichstein, *British Pat.* 466,548 (1937); R. Pasternack and G. O. Cragwall, *U. S. Pat.* 2,185,383 (1940).

(22) W. N. Haworth, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 710 (1938).

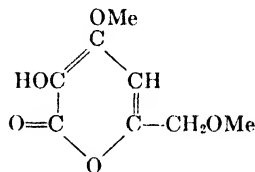
with sodium methoxide in methyl alcohol and lactonization, which cannot take place between C1 and C4 as in methyl 2-keto-D-gluconate (XXXVII), is brought about between C1 and C5 giving a  $\delta$ -lactone ring. Simultaneously with lactonization the expected enolization proceeds to give the enediolic system at C2 and C3 together with the elimination of the elements of methyl alcohol between C4 and C5. There is thus obtained the six-membered ring analog of L-ascorbic acid (XLII), an  $\alpha$ -pyrone derivative resembling in its properties the 3-methyl derivatives of D-araboascorbic acid and L-ascorbic acid.



Maltol



Hexenolactone



XLII

It is worthy of note that the substance maltol, 2-methyl-3-hydroxy- $\gamma$ -pyrone, which is related to XLII, occurs in pine needles<sup>22a</sup> along with L-ascorbic acid. This substance maltol, however, is not a growth promoter like L-ascorbic acid but an inhibitor, a point of interest inasmuch as it has been obtained in considerable quantity by the degradation of streptomycin.<sup>22b</sup> Since the naturally occurring para-ascorbic acid or hexenolactone,<sup>22c, 22d</sup> also a derivative of  $\alpha$ -pyrone, is known to display anti-growth properties,<sup>22e</sup> it is not unlikely that other analogs of ascorbic acid may be growth inhibitors.

### 3. Condensation of Hydroxy Aldehydes with Ethyl Glyoxylate or Ethyl Mesoxalate

This method has an analogy in the well known acyloin condensation, a reaction which takes place between two molecules of an aromatic aldehyde in a solution containing an alkali cyanide. Thus for example, benzaldehyde gives rise to benzoin, a compound in which the enediolic system,  $\text{—C(OH)=C(OH)—}$ , exists mainly in the ketonic form  $\text{—CO—CHOH—}$ . If a hydroxy aldehyde like D-glucose (X) is allowed to

(22a) W. Feuerstein, *Ber.*, **34**, 1804 (1901).

(22b) J. R. Schenck and M. A. Spielman, *J. Am. Chem. Soc.*, **67**, 2276 (1945).

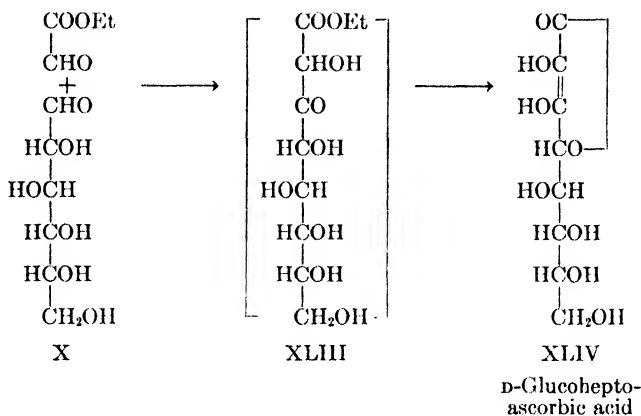
(22c) R. Kuhn and D. Jerchel, *Ber.*, **76B**, 413 (1943).

(22d) P. B. Medawar, G. M. Robinson and R. Robinson, *Nature*, **151**, 195 (1943).

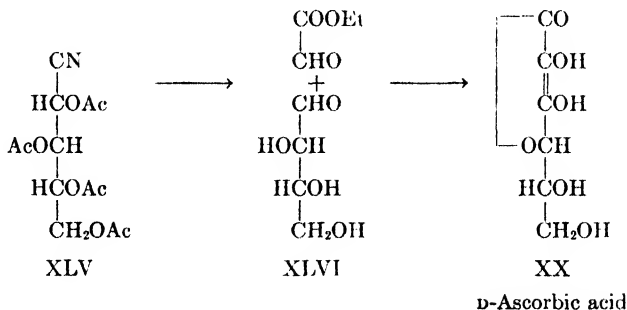
(22e) T. Hauschka, G. Toennies, and A. Swain, *Science*, **101**, 383 (1945).



react with ethyl glyoxylate (prepared from ethyl tartrate by oxidation with lead tetraacetate<sup>23</sup>) under the influence of sodium methoxide a reaction analogous to the benzoin change proceeds. The intermediate product (XLIII) is not isolated, instead there is obtained as the final



stage D-glucoheptoascorbic acid (XLIV).<sup>24</sup> This reaction is capable of wide application and numerous analogs of ascorbic acid may be prepared thereby. The acetylated sugar acid nitriles can replace the free sugars in this reaction since they yield upon treatment with alkali an aldehyde with one less carbon atom. Thus the reaction between either tetraacetyl-D-xylononitrile (D-threose cyanohydrin tetraacetate, XLV); or between D-threose (XLVI), and ethyl glyoxylate, affords D-ascorbic acid (XX).



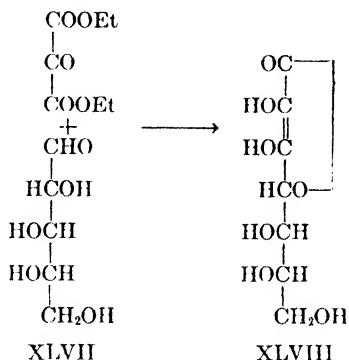
Furthermore it has been shown that ethyl mesoxalate can be used instead

(23) R. Criegie, L. Kraft and B. Rank, *Ann.*, **507**, 159 (1933).

(24) B. Helferich and O. Peters, *Ber.*, **70B**, 465 (1937).

(25) B. Helferich, German Pat. 683,954 (1939).

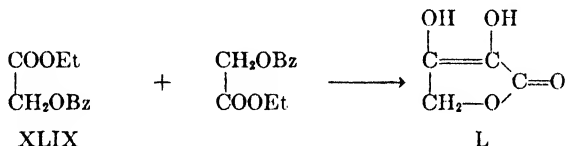
of ethyl glyoxylate,<sup>25</sup> L-arabinose (XLVII) for example reacting with ethyl mesoxalate to give L-glucoascorbic acid (XLVIII).<sup>26</sup>



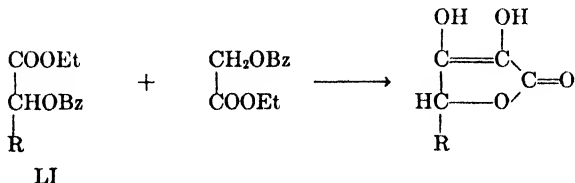
This is not an efficient method for the preparation of synthetic Vitamin C owing to the difficulty of obtaining L-threose.<sup>26</sup>

#### 4. Condensation of Esters of Hydroxy Acids

This method, analogous to the well-known Claisen condensation, can be illustrated by reference to the condensation of two molecules of ethyl benzoyloxyacetate (XLIX) under the influence of potassium.<sup>27</sup>



There is obtained the hydroxytetrone acid L, the simplest analog of ascorbic acid containing the characteristic five-membered ring. Although this compound shows many of the reactions of an ascorbic acid it exhibits no antiscorbutic activity. If instead of using only ethyl benzoyl-



(26) B. Helferich, U. S. Pat. 2,207,680 (1940).

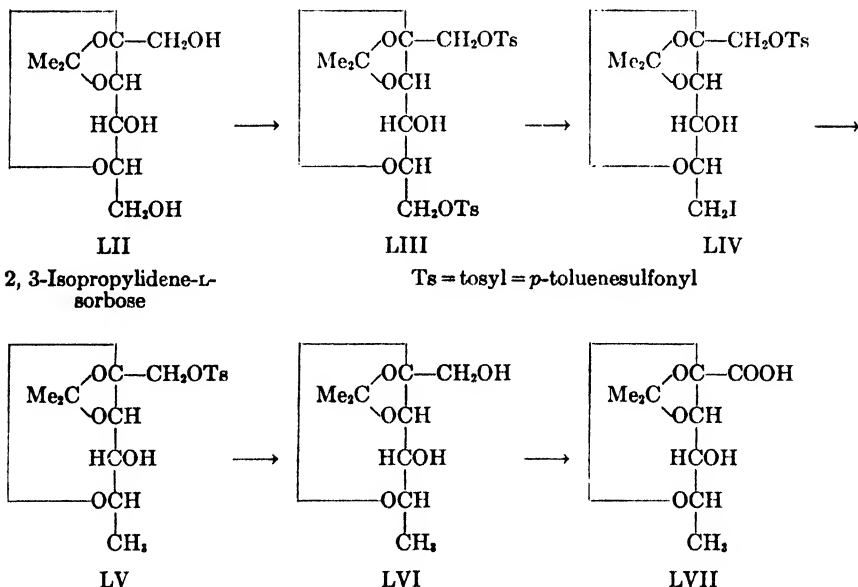
(27) F. Micheel and F. Jung, *Ber.*, **66B**, 1291 (1933).

(28) F. Micheel and H. Haarkoff, *Ann.*, **545**, 28 (1940).

oxyacetate in this reaction one uses a mixture of ethyl benzyloxyacetate and the benzoyl derivative of a polyhydroxy ester LI, a substituted hydroxytetronic acid or analog of ascorbic acid can be produced.<sup>28</sup>

## II. DESOXY AND AMINO DERIVATIVES OF ASCORBIC ACID

The variation in the antiscorbutic activity displayed by the various analogs of L-ascorbic acid makes it abundantly clear that the activity is dependent upon the stereochemical configuration of the molecule as a whole, and it would appear that the more closely the structure of a particular analog approaches that of the natural Vitamin C the greater will be the antiscorbutic power. Support for this view is illustrated by 6-deoxy-L-ascorbic acid which is obtained from L-sorbose.<sup>29,30</sup> Condensation of L-sorbose with acetone gives a mixture of 2,3-isopropylidene-L-sorbose (LII) and the diisopropylidene derivative. Treatment of LII with *p*-toluenesulfonyl chloride yields 1,6-ditosyl-2,3-isopropylidene-L-sorbose (LIII). The greater reactivity of the tosyl group at C6 enables

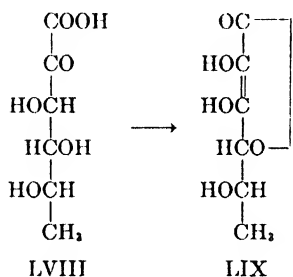


it to be replaced by iodine through the agency of sodium iodide. The 1-tosyl-6-iodo-2,3-isopropylidene-L-sorbose (LIV) thus obtained affords upon catalytic hydrogenation with Raney nickel in alkaline solution

(29) H. Müller and T. Reichstein, *Helv. Chim. Acta*, **21**, 273 (1938).

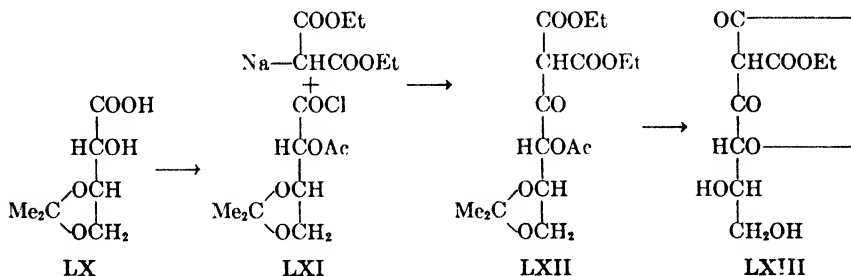
(30) F. Hoffmann-La Roche & Co. A.-G., Swiss Pat. 203,549 (1939).

6-desoxy-1-tosyl-2,3-isopropylidene-L-sorbose (LV). When the latter is allowed to react with sodium amalgam in aqueous alcohol, elimination of the tosyl group takes place and there is produced 2,3-isopropylidene-6-desoxy-L-sorbose (LVI). Oxidation of LVI with potassium permanganate converts the free primary alcoholic group at C1 into a carboxyl group giving LVII. Hydrolysis of this compound with acid ethyl alcohol causes simultaneous elimination of the acetone group and formation of 6-desoxy-L-ascorbic acid (LIX).



6-Desoxy-L-ascorbic acid

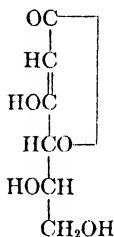
High antiscorbutic power is also reported to be shown by a derivative of L-ascorbic acid in which the enolic hydroxyl group at C2 is replaced by an amino group. 3,4-Isopropylidene-L-threonic acid (LX), prepared from 5,6-isopropylidene-L-ascorbic acid, is converted into the 2-acetyl-3,4-isopropylidene-L-threonyl chloride (LXI) and this is then allowed to react with the sodium derivative of ethyl malonate.



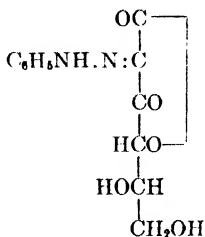
Hydrolysis of the condensate gives LXIII and this by the agency of methyl alcoholic alkali affords the enol form of 2-desoxy-L-ascorbic acid (LXIV).<sup>31</sup> When the latter is allowed to react with benzene diazonium chloride there is formed the mono(phenylhydrazone) LXV from which

(31) F. Micheel and K. Hasse, *Ber.*, 69B, 879 (1936).

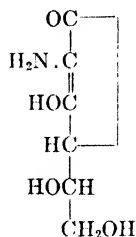
the 2-amino analog (LXVI) of L-ascorbic acid is derived by catalytic reduction.<sup>32</sup> Similarly, catalytic reduction of the normal di(phenylhydrazine) LXVIII of dehydro-L-ascorbic acid (LXVII) gives rise to the 2,3-diamino derivative LXIX.<sup>33</sup>



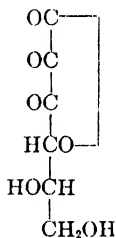
LXIV



LXV

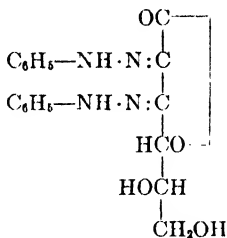


LXVI

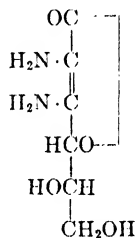


LXVII

Dehydro-L-  
ascorbic acid



LXVIII



LXIX

Both the monoamino and the diamino analogs of ascorbic acid exhibit powerful reducing properties, and the monoamino compound has a high antiscorbutic activity, probably due to the fact that it undergoes conversion to L-ascorbic acid *in vivo* by a process of deamination.

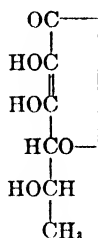
### III. PHYSIOLOGICAL ACTIVITY OF ANALOGS OF ASCORBIC ACID

The methods referred to above have enabled a number of analogs of ascorbic acid to be isolated. These have been tested for antiscorbutic activity and the simple rule emerges that the five-atom lactone ring containing the enediolic system is essential for activity, and furthermore this lactone ring must be on the right side of the chain of carbon atoms when

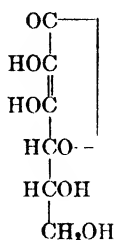
(32) F. Micheel and R. Mittag, *Naturwissenschaften*, **25**, 158 (1937); *Z. physiol. Chem.*, **247**, 34 (1937).

(33) F. Micheel, G. Bode and R. Siebert, *Ber.*, **70B**, 1862 (1937).

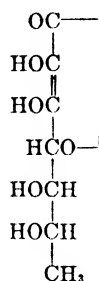
the formula is written according to the Fischer convention.<sup>32</sup> This can be demonstrated by reference to the formulas below.



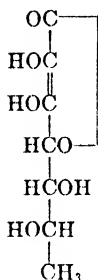
LIX  
6-Desoxy-L-  
ascorbic acid  
 $\left(\frac{1}{3}\right)$



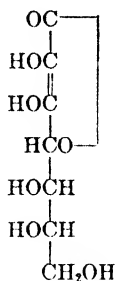
XXXIX  
D-Arabo-  
ascorbic acid  
 $\left(\frac{1}{20}\right)$



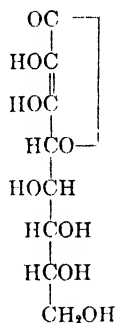
LXX  
L-Rhamno-  
ascorbic acid  
 $\left(\frac{1}{5}\right)$



LXXI  
L-Fuco-  
ascorbic acid  
 $\left(\frac{1}{50}\right)$



XLVIII  
L-Gluco-  
ascorbic acid  
 $\left(\frac{1}{40}\right)$



XLIV  
D-Glucohepto-  
ascorbic acid  
 $\left(\frac{1}{100}\right)$

Active analogs of ascorbic acid.<sup>34-37</sup>

(The activity shown in brackets is based on unit activity for L-ascorbic acid.)

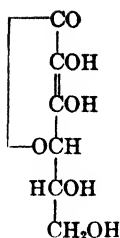
The substances reductone (LXXVII; obtainable from sugars by the

(34) V. Demole, *Biochem. J.*, **28**, 770 (1934).

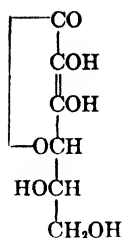
(35) O. Dalmer and T. Moll, *Z. physiol. Chem.*, **222**, 116 (1933).

(36) T. Reichstein, L. Schwartz and A. Grüssner, *Helv. Chim. Acta*, **18**, 353 (1935).

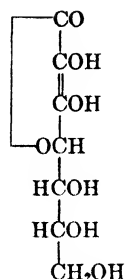
(37) V. Demole, *Helv. Chim. Acta*, **21**, 277 (1938).



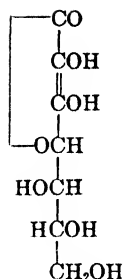
XX  
D-Xylo-  
ascorbic acid



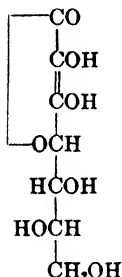
LXXII  
L-Arabo-  
ascorbic acid



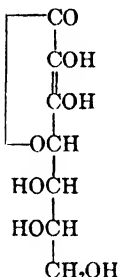
XV  
D-Gluco-  
ascorbic acid



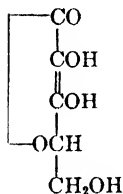
LXXIII  
D-Galacto-  
ascorbic acid



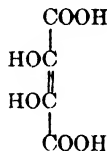
LXXIV  
L-Gulo-  
ascorbic acid



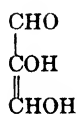
LXXV  
L-Allo-  
ascorbic acid



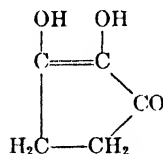
XXXVI  
L-Erythro-  
ascorbic acid



LXXVI



LXXVII



LXXVIII

Inactive analogs of L-ascorbic acid.<sup>3a, 34, 35</sup>

action of alkali<sup>39</sup>) and dihydroxymaleic acid (LXXVI) resemble L-ascorbic acid in their reducing properties but they have no antiscorbutic power. Similarly, reductic acid (LXXVIII), which can be prepared from D-glucuronic acid, pectic acid and other carbohydrates by heating them with dilute mineral acid,<sup>30, 40</sup> is inactive. Reductic acid undergoes

(38) S. S. Zilva, *Biochem. J.*, **29**, 1612 (1935).

(39) H. von Euler and C. Martius, *Svensk Kem. Tid.*, **45**, 73 (1933).

(40) T. Reichstein and R. Oppenauer, *Helv. Chim. Acta*, **16**, 988 (1933).

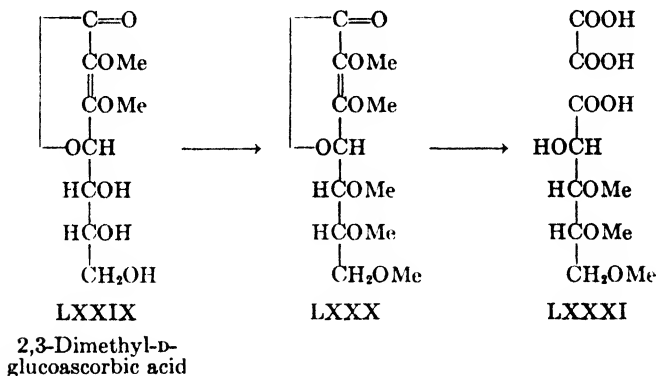
the reversible oxidation characteristic of L-ascorbic acid with iodine in aqueous solution.

#### IV. STRUCTURE OF ANALOGS OF ASCORBIC ACID

From the method of preparation of the analogs of L-ascorbic acid and from their close resemblance to L-ascorbic acid in respect to chemical properties and absorption data, all these derivatives were assumed by analogy to have the characteristic unsaturated five-membered ring system already proved to be present in L-ascorbic acid. In two cases, namely that of D-glucos- (XV) and D-araboascorbic acid (XXXIX) the assumption has proved correct.

##### 1. D-Glucoascorbic Acid

Treatment of D-glucoascorbic acid (XV) with diazomethane gives a 2,3-dimethyl derivative (LXXIX) and this upon repeated treatment with silver oxide and methyl iodide yields 2,3,5,6,7-pentamethyl-D-glucoascorbic acid (LXXX). Ozonization of the latter followed by hydrolysis gives oxalic acid and 3,4,5-trimethyl-D-arabonic acid (LXXXI). This acid was shown to possess a free hydroxyl group at C2 by reason of the fact that the amide of LXXXI gives a positive Weerman reaction for  $\alpha$ -hydroxy amides, i.e., when the amide is treated with sodium hypochlorite, sodium isocyanate is produced, the latter being identified by



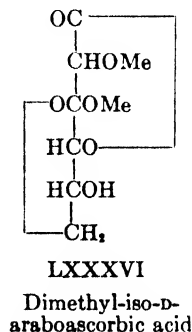
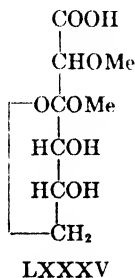
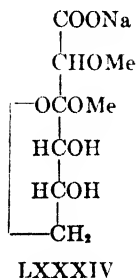
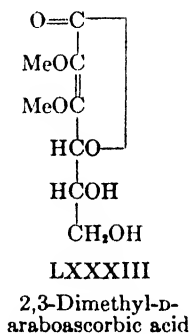
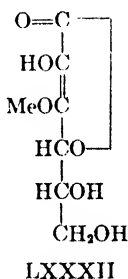
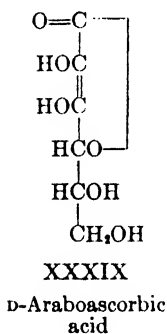
its reaction with semicarbazide hydrochloride which forms the characteristic crystalline hydrazo-dicarbonamide. The location of a hydroxyl group at C2 in the trimethyl-D-arabonic acid (LXXXI) shows that C2 in the latter (or C4 in the original 2,3,5,6,7-pentamethyl-D-glucoascorbic acid (LXXX)) must be the carbon atom engaged in the lactone ring. It follows therefore that the lactone ring is of the five-



atom type and since ozonolysis gives oxalic acid it is clear that the double bond is situated between C2 and C3. The structure LXXX assigned to pentamethyl-D-glucoscorbic acid is thus correct and since no structural change takes place during the conversion of D-glucoscorbic acid into the pentamethyl derivative LXXX, as shown by the similarity of the absorption bands displayed by XV, LXXIX and LXXX, it is concluded that D-glucoscorbic acid has the structure XV.<sup>43</sup>

## 2. D-Araboascorbic Acid

There is good evidence for the presence of a five-membered ring in D-arboascorbic acid (XXXIX). This analog of L-ascorbic acid, prepared by method 2 (page 87) from methyl 2-keto-D-gluconate shows the same chemical properties and the same absorption characteristics as L-ascorbic acid. Titration of XXXIX with diazomethane affords, as in the case of L-ascorbic acid, a 3-methyl derivative LXXXII which upon further action with diazomethane gives rise to the 2,3-dimethyl-D-arboascorbic acid (LXXXIII).<sup>20, 44</sup>

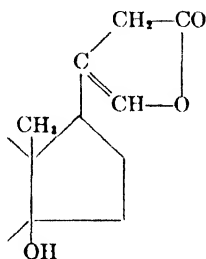


(43) W. N. Haworth, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 549 (1937).

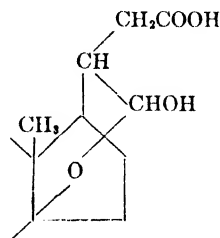
(44) E. G. E. Hawkins, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 246 (1939).

This 2,3-dimethyl-D-araboascorbic acid (LXXXIII) behaves in precisely the same way as 2,3-dimethyl-L-ascorbic acid when treated with alkaline reagents.<sup>45</sup> Saturation of the double bond between C2 and C3 by ring closure between C6 and C3 and transference of a proton to C2 takes place, giving LXXXVI.

This saturation of a double bond is analogous to the isomerism effected in the steroid aglycons, for when the latter are treated with alkali the system LXXXVII is transformed into LXXXVIII.<sup>46</sup>

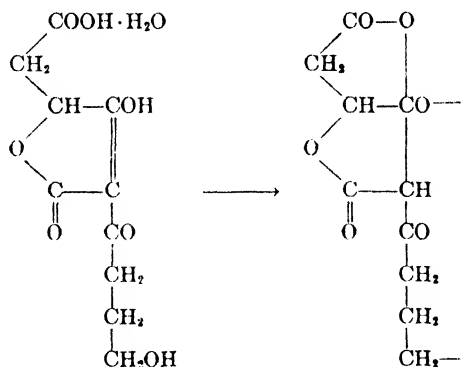


LXXXVII



LXXXVIII

In this connection there should also be mentioned the formation of carlic acid (XC) from LXXXIX (formed from D-glucose as a metabolic product of the mold *Penicillium charlesii* G. Smith<sup>47</sup>).



LXXXIX

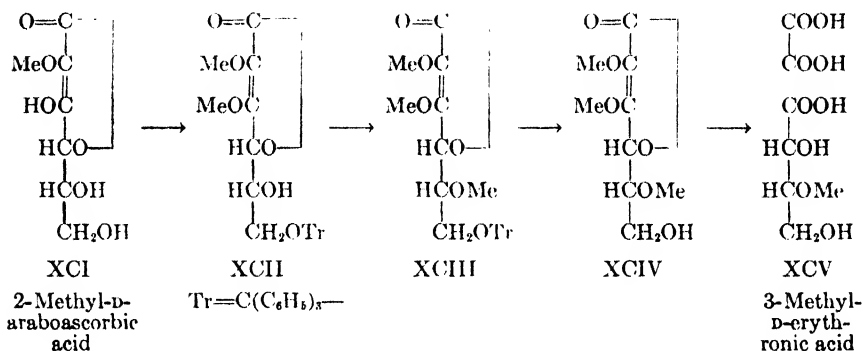
XC  
Carlic acid

(45) W. N. Haworth, E. L. Hirst, F. Smith and W. J. Wilson, *J. Chem. Soc.*, 829 (1937).

(46) W. A. Jacobs and E. L. Gustus, *J. Biol. Chem.*, **74**, 811 (1927); R. C. Elderfield, *Chem. Revs.*, **17**, 208 (1935).

(47) P. W. Clutterbuck, H. Raistrick and F. Reuter, *Biochem. J.*, **29**, 300, 871, 1300 (1935); R. W. Herbert and E. L. Hirst, *ibid.*, **29**, 1881 (1935).

The lactone ring which engaged C1 and C4 in LXXXVI is a normal type of lactone and opens giving the simple salt LXXXIV (compare with the original D-araboascorbic acid or L-ascorbic acid, which form monobasic salts by loss of a proton from C3 and which do not show any tendency to form open chain compounds). Acidification of an alkaline solution of LXXXIV does not give the free acid LXXXV but instead lactonization takes place and there is formed the dimethyl-iso-D-araboascorbic acid (LXXXVI).<sup>48</sup> This substance contains an interlocking pair of 5-atom rings, a structural system which is often very stable (compare D-mannosaccharo-1,4:3,6-dilactone) and one which is readily formed when conditions are favorable (compare the glucosides and lactones of 3,6-anhydro-D-glucose<sup>48</sup>).



The analogy between D-araboascorbic acid and L-ascorbic acid can be traced still further by the observation that treatment of the dimethyl-iso-D-araboascorbic acid (LXXXVI) with methyl alcoholic hydrogen chloride produces 2-methyl-D-araboascorbic acid (XCI).

That the dimethyl-D-araboascorbic acid (LXXXIII) still contains two hydroxyl groups, one a primary alcoholic and the other a secondary alcoholic grouping, is shown by the observation that it affords a 6-trityl derivative XCII from which by methylation with silver oxide and methyl iodide there is formed 6-trityl-2,3,5-trimethyl-D-araboascorbic acid (XCIII). Removal of the trityl group with hydrogen chloride yields 2,3,5-trimethyl-D-araboascorbic acid (XCIV) which undergoes smooth ozonolysis. Decomposition of the ozonide with water produces oxalic acid and 3-methyl-D-erythronic acid (XCV) the latter being shown to have a hydroxyl group at C2 by the fact that the amide of the acid (XCV) gives a positive Weerman test for α-hydroxy amides. The location of this hydroxyl group at C2 in XCV together with the formation of XCV

and oxalic acid during ozonolysis establishes in this second analog the presence of the characteristic unsaturated five-membered ring of L-ascorbic acid.<sup>20</sup>

There is little doubt therefore that all the true analogs of L-ascorbic acid discussed above contain the unsaturated five atom ring system to which are attached two enolic hydroxyl groups on the carbon atoms of the double bond.

## V. ANALOGS OF L-ASCORBIC ACID CONTAINING ONE ENOLIC HYDROXYL GROUP

### 1. From D-Mannosaccharo-1,4:3,6-dilactone

Simultaneously with the investigations upon the natural L-ascorbic acid and its analogs, an examination was made of the only other known acidic substance in the sugar series which displayed unusual reducing properties. This substance, D-mannosaccharo-1,4:3,6-dilactone, has been known for many years to have the unexpected power of reducing Fehling's solution,<sup>49, 50</sup> but until recently an explanation for this curious phenomenon based on experimental facts was lacking (cf. ref. 51). Early in the investigations it was recognized that the reducing power became manifest only after treatment with alkaline reagents, a treatment which also resulted in the production of a substance in solution showing a strong selective absorption band at 2630 Å moving upon acidification to 2290 Å<sup>52, 53</sup> (cf. with L-ascorbic acid which shows a band at 2650 Å in dilute sodium hydroxide moving to 2450 Å upon addition of acid). Moreover the acidified solutions containing the reactive substance showed a close resemblance in this respect to solutions of L-ascorbic acid and its analogs. It was therefore considered likely that the dilactone was undergoing isomerization and enolization to give a substance analogous to L-ascorbic acid. This has proved to be the case, for careful control of the treatment of D-mannosaccharo-1,4:3,6-dilactone (XCVI) with sodium methoxide in methyl alcohol gives rise to the crystalline compound XCVII. The latter, having a formula  $C_6H_6O_6$  is isomeric with the dilactone XCVI. The enol XCVII reacts with chlorine, bromine, and potassium permanganate in acid solution. It reduces Fehling's solution on warming but unlike L-ascorbic acid it will not reduce silver nitrate solution. It also differs from L-ascorbic acid in that it reacts in acid solution

(49) H. Kiliani, *Ber.*, 20, 2710 (1887).

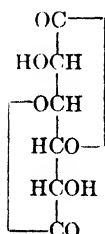
(50) E. Fischer, *Ber.*, 24, 539 (1891).

(51) K. Rehorst, *Ber.*, 71B, 923 (1938).

(52) F. Smith, *Chemistry & Industry*, 57, 450 (1938).

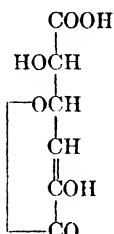
(53) W. N. Haworth, Doreen Heslop and F. Smith, *J. Chem. Soc.*, 217 (1944).

with only a small amount of iodine (L-ascorbic acid reacts with two atomic proportions) and a closer examination of the reaction of XCVII with chlorine has demonstrated that it reacts with four atomic proportions while L-ascorbic acid and its analogs react with two atomic proportions under

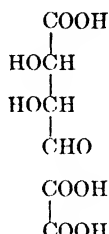


XCVI

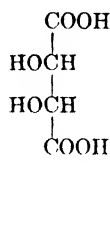
D-Mannosaccharo-  
1,4:3,6-dilactone



XCVII

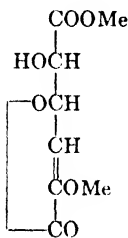


XCVIII

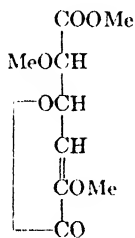


XCIX

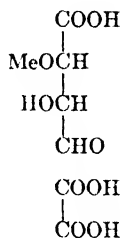
*meso*-Tartaric  
acid



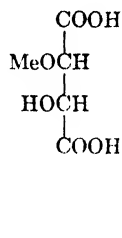
C



CI

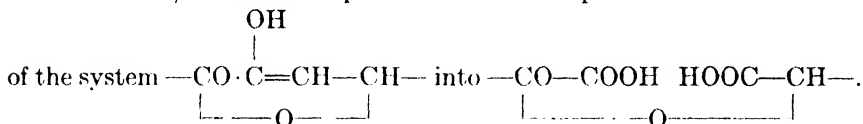


CII



CIII

the same conditions. The unsaturated five-membered ring system remains intact in the presence of alkali when two protons are set free, one from the carboxyl group at C1 and one from the enolic hydroxyl group at C5. In alkaline solution the enol reacts with six atomic proportions of iodine, an oxidation process which corresponds to the conversion



When the enol (XCVII) of 5-keto-4-desoxy-mannosaccharo-3,6-lactone is subjected to ozonolysis, cleavage occurs at the double bond with the formation of oxalic acid and D-erythronic acid (XCVIII), the latter being identified by the fact that upon oxidation with bromine it yields *meso*-tartaric acid (XCIX). The formation of oxalic acid and erythronic acid locates the double bond in XCVII between C4 and C5 and demonstrates that C4 carries a hydrogen atom while C5 has attached to

it the enolic hydroxyl group. If the reverse were the case, ozonolysis would have provided glyoxylic acid and *meso*-tartaric acid.<sup>54</sup> Treatment of the enol XCVII with ethereal diazomethane effects esterification at C1 and the introduction of a methyl group into the enolic hydroxyl at C5 and there is formed 5-methyl- $\Delta^4$ -D-mannosaccharo-3,6-lactone methyl ester (C). Completion of the methylation of this substance with silver oxide and methyl iodide yields 2,5-dimethyl- $\Delta^4$ -D-mannosaccharo-3,6-lactone methyl ester (CI). Ozonization of the latter provides oxalic acid and 3-methyl-D-erythronic acid (CII) identified by its transformation with bromine into D-erythro-2-hydroxy-3-methoxy-succinic acid (CIII).<sup>20</sup> The identification of the latter proves that the methyl derivative contains a five-membered ring which engages C6 and C3. Since no structural change takes place during the conversion of the enol XCVII into this methyl derivative CI, it follows that the former also contains the same five-membered ring as shown in the formula XCVII assigned to it. This enol of 5-keto-4-desoxy-D-mannosaccharo-3,6-lactone is therefore the substance responsible for the curious reducing phenomenon displayed by D-mannosaccharodilactone.<sup>53</sup>

The isomerization of D-mannosaccharodilactone which proceeds under the influence of alkaline reagents can also be brought about by diazomethane and by silver oxide and methyl iodide. In both cases isomerization is accompanied by methylation and there results 2,5-dimethyl- $\Delta^4$ -D-mannosaccharo-3,6-lactone methyl ester (CI).<sup>54</sup>

## 2. From D-Glucosaccharic Acid and its Derivatives

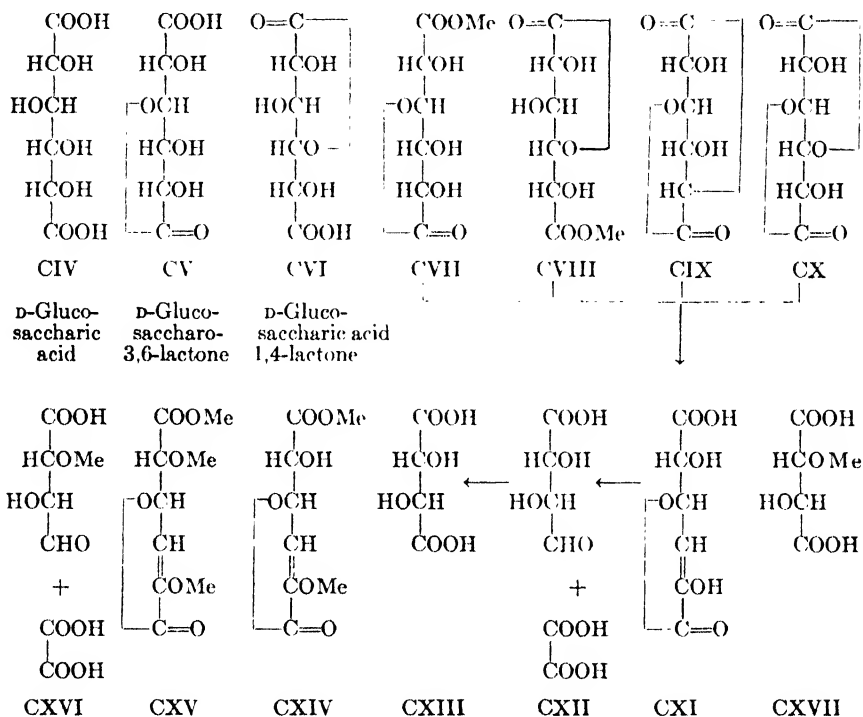
The peculiar isomerization outlined above giving rise to analogs of ascorbic acid is not confined to D-mannosaccharo-dilactone for it has been shown that the two dilactones CIX and CX of D-glucosaccharic acid (CIV), prepared from D-glucosaccharo-3,6-lactone (CV) and D-glucosaccharo-1,4-lactone (CVI), respectively,<sup>54,55</sup> behave in a similar fashion and curiously enough the ester lactones CVII and CVIII will undergo the same isomerization when subjected to the influence of alkaline reagents.<sup>55,56</sup> The reducing properties displayed by the two dilactones CIX and CX and the two ester lactones CVII and CVIII in alkaline solution or in acid solution after pretreatment with alkali has been traced to the formation in each case of the enolic substance CXI. The isomerization is best effected with methyl alcoholic sodium methoxide and from CVII, CVIII, CIX and CX it has been possible to isolate the

(54) Doreen Heslop and F. Smith, *J. Chem. Soc.*, 577 (1944).

(55) F. Smith, *J. Chem. Soc.*, 633 (1944).

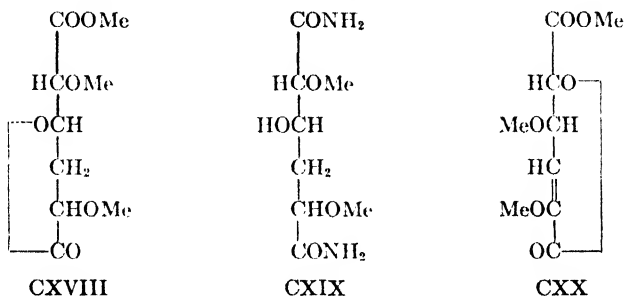
(56) Doreen Heslop and F. Smith, *J. Chem. Soc.*, 637 (1944).

enol CXI in crystalline form. The properties of this compound are almost identical with those shown by the corresponding enol XCVII from *D*-mannosaccharodilactone (XCVI). The structure of CXI has been established as a result of the following series of experiments. Ozonization of the enol CXI furnishes oxalic acid and *L*-threuronic acid (CXII), the latter being identified by its oxidation with bromine to



*L*-threo-dihydroxy-succinic acid (*L*(*dextro*)-tartaric acid, CXIII). This result establishes the position of the double bond between C4 and C5 and demonstrates that C4 carries only one hydrogen atom while C5 has attached to it the enolic hydroxyl group. Treatment of the enol CXI with ethereal diazomethane gives 5-methyl- $\Delta^4$ -*D*-glucosaccharo-3,6-lactone methyl ester (CXIV) which upon further methylation with silver oxide and methyl iodide yields 2,5-dimethyl- $\Delta^4$ -*D*-glucosaccharo-3,6-lactone methyl ester (CXV). When the latter is subjected to ozonolysis there is formed oxalic acid and 3-methyl-*L*-threuronic acid (CXVI). Oxidation of this aldehydic acid (CXVI) with bromine gives rise to a monomethyl derivative (CXVII) of *L*-threo-dihydroxy-succinic acid.

These facts confirm that the double bond is between C4 and C5 and that these carbon atoms carry one —H and one —OH respectively. These facts, however, do not enable the position of the lactone ring to be fixed because it will be apparent that the same acid (CXVII) would arise from the structure CXV and from the structure CXX containing a



six-membered lactone ring engaging C6 and C2. The problem was solved as follows. Catalytic hydrogenation of the unsaturated ester CXV affords the desoxy compound CXVIII which was smoothly transformed into the crystalline diamide CXIX. Since this diamide displayed a negative Weerman reaction for  $\alpha$ -hydroxy amides it follows that the hydroxyl group exposed during the conversion of the desoxy ester CXVIII into the desoxy amide CXIX must be attached to C3 and not to C2. Hence the lactone ring of the desoxy ester CXVIII and therefore of CXV and the original enol CXI, must engage C6 and C3.<sup>57</sup> Additional evidence is forthcoming from the observation that opening of the lactone ring of the desoxy lactone effects a rotation change in the negative sense. By Hudson's lactone rule this would suggest that the lactone ring engages C6 and C3, for the opening of a lactone ring engaging C6 and C2 would be expected to result in a change of rotation in a positive direction.<sup>58</sup>

The formation of this analog CXI of L-ascorbic acid containing one enolic hydroxy group is therefore responsible for the reducing properties displayed by the two dilactones CIX and CX and the two ester lactones CVII and CVIII of D-glucosaccharic acid.

The methylated analog CXV of L-ascorbic acid, 2,5-dimethyl- $\Delta^4$ -D-glucosaccharo-3,6-lactone methyl ester has been obtained by simultaneous enolization and methylation of a number of substances. For instance it is derivable by treatment, with silver oxide and methyl iodide, of D-glucosaccharo-1,5:3,6-dilactone (CIX), D-glucosaccharo-1,4:

(57) F. Smith, *J. Chem. Soc.*, 510 (1944).

(58) O. T. Schmidt, H. Dippold and H. Zeiser, *Ber.*, 70B, 2402 (1937).



3,6-dilactone (CX), D-glucosaccharo-1,4-lactone methyl ester (CVIII), D-glucosaccharo-3,6-lactone methyl ester (CVII), D-glucosaccharo-1,4-lactone (CVI), D-glucosaccharo-3,6-lactone (CV) and from silver D-glucosaccharate.<sup>57, 58</sup> It can also be produced by the action of diazomethane upon D-glucosaccharic acid (CIV),<sup>58</sup> the two monolactones of D-glucosaccharic acid (CV and CVI) and the dilactone CX. The methylated unsaturated compound was first isolated but not identified by Pryde and Williams<sup>59</sup> who obtained it as a by-product during the methylation of D-glucurone (D-glucuronic acid lactone) with silver oxide and methyl iodide, in which reaction oxidation precedes the simultaneous enolization and methylation.<sup>60</sup>

For the production of these analogs of ascorbic acid from D-glucosaccharic acid and its derivatives it is necessary for both carboxyl groups to be esterified either internally as in the dilactones CIX and CX, or partly internally and partly externally as with the ester lactones CVII and CVIII. D-Glucosaccharic acid (CIV) and the two monolactones CV and CVI which have free carboxyl groups do not show any reducing activity in alkaline solution whereas ethyl D-glucosaccharate does show reducing power though to a much smaller extent. It would appear therefore that the electronic influence of both carbonyl groups at C1 and C6 is essential for the formation of the unsaturated five-membered ring characteristic of L-ascorbic acid. When the >CO groups exist in carboxyl ions the isomerization cannot be brought about, probably be-

cause the resonance in this group,  $\left. \begin{array}{c} \text{O} \\ \diagup \\ \text{—C} \\ \diagdown \\ \text{O} \end{array} \right\} \text{H}$ , masks the true carbonyl

properties. In view of the fact that the methyl iodide, silver oxide and the diazomethane methods, which also produce this peculiar isomerization, probably effect esterification in the early stages of the reaction, it is not unlikely that the two carbonyl groups play a similar and important part during the production of 2,5-dimethyl- $\Delta^4$ -D-glucosaccharo-3,6-lactone methyl ester from D-glucosaccharic acid and its derivatives.

(59) J. Pryde and R. T. Williams, *Biochem. J.*, **27**, 1205 (1933).

(60) F. Smith, *J. Chem. Soc.*, 584 (1944).

# SYNTHESIS OF HEXITOLS AND PENTITOLS FROM UNSATURATED POLYHYDRIC ALCOHOLS

BY R. LESPIEAU

*École Normale Supérieure, Paris, France*

Translated from the French by Donald and Gertrude Hoffman,  
The Ohio State University, Columbus, Ohio

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## I. HISTORICAL FOREWORD

The syntheses of certain hexitols and pentitols that are reviewed in the present article were begun about 1928. Their origin is related to an investigation made by G. Griner thirty-six years previously, the essential results of which will now be described.

Griner,<sup>1</sup> in hydrogenating acrolein by means of a zinc-copper couple and acetic acid, obtained a liquid corresponding in properties and analysis to the dimolecular reduction product, divinylglycol,  $\text{CH}_2=\text{CH}-\text{CHOH}-\text{CHOH}-\text{CH}=\text{CH}_2$ . Preparations involving acrolein were difficult to carry out at that time since no commercial acrolein was available and the acrolein prepared in the laboratory resinified very rapidly. This divinylglycol, like tartaric acid, may exist in active (*d* or *l*), meso, or racemic (*d, l*), forms, as Griner well knew. If the *d, l*-mixture were present Griner reasoned that preferential attack by a suitable mold might induce optical activity, and he requested LeBel to attempt such cultures. LeBel's experiments were successful, but optical activity did not appear, and Griner concluded that he had only the meso form of divinylglycol. However, this conclusion was erroneous for it has been found that divinylglycol prepared according to Griner's procedure contains both the meso and racemic forms of the compound.

It is known that a vinyl group ( $\text{CH}_2=\text{CH}-$ ) may be oxidized to the corresponding diol ( $\text{CH}_2\text{OH}-\text{CHOH}-$ ) with dilute potassium permanganate; in the case of Griner's divinylglycol such oxidation might lead to a hexitol, from which it might have been possible to proceed to an aldose. However, in spite of all efforts to moderate the action of permanganate, it was not possible to stop the oxidation at the desired stage and much of the starting material was destroyed. Griner then added two moles of hypochlorous acid to his divinylglycol and thus obtained two stereoisomeric forms of divinylglycol dichlorohydrin ( $\text{CH}_2\text{Cl}-\text{CHOH}-\text{CHOH}-\text{CHOH}-\text{CHOH}-\text{CH}_2\text{Cl}$ ). Concerning the conversion of these dichlorohydrins to the corresponding hexitols, he published only the following lines:

"J'ajouterais, en outre, que j'ai réussi, au moyen de la seconde, à remonter à la mannite par un procédé qui sera décrit plus tard et qu'il m'a été impossible d'obtenir, par la même méthode, l'alcool hexatomique correspondant à la première." \*

Griner published nothing more on this subject.

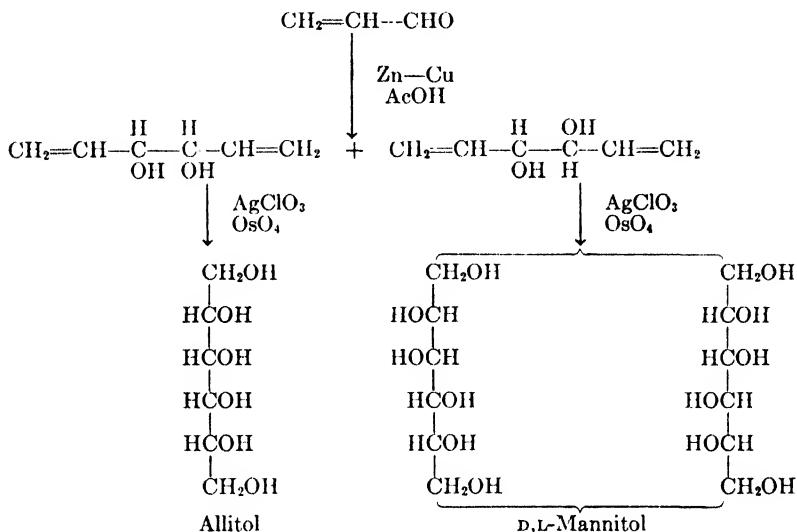
(1) G. Griner, *Ann. chim. phys.*, [6] 26, 369 (1892).

\* I will add that I have succeeded in obtaining mannitol from the second (divinylglycol dichlorohydrin) by means of a procedure which will be described later, but that it has been impossible for me to obtain the hexitol corresponding to the first (dichlorohydrin) by the same method.

## II. SYNTHESIS OF HEXITOLS FROM UNSATURATED INTERMEDIATES

1. *Methods of Preparation*

Very little was published on the synthesis of hexitols from unsaturated intermediates between the time of Griner's work and 1933, when it occurred to me that it might be possible to add four hydroxyl groups to divinylglycol by means of a solution of silver chlorate containing a small amount of osmic acid. In carrying out this work I had the assistance of one of my students, Joseph Wiemann. We succeeded far beyond our expectations and obtained allitol, unknown up to that time, and D,L-mannitol.



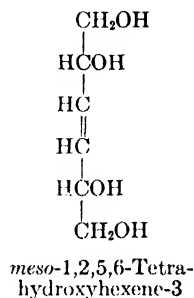
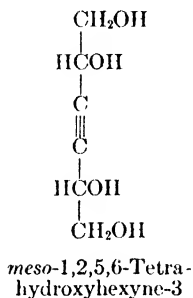
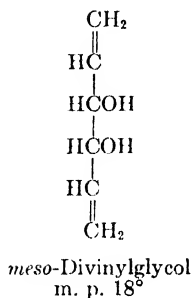
Another method of synthesis was also used. This involved the action of chloroacetaldehyde on the Grignard reagent derived from acetylene in order to obtain the meso divinylacetylene dichlorohydrin,  $\text{CH}_2\text{Cl}-\text{CHOH}-\text{C}\equiv\text{C}-\text{CHOH}-\text{CH}_2\text{Cl}$ ,<sup>2</sup> from which one passed to the corresponding hexynetetrol,  $\text{CH}_2\text{OH}-\text{CHOH}-\text{C}\equiv\text{C}-\text{CHOH}-\text{CH}_2\text{OH}$ . This, in turn, was reduced to the hexenetetrol,  $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}=\text{CH}-\text{CHOH}-\text{CH}_2\text{OH}$ , by means of Bourguel's catalyst,<sup>3</sup> a dispersion of colloidal palladium on starch. When the hexenetetrol was hydroxylated by the use of silver chlorate and osmic acid, two hexitols, dulcitol and allitol, were obtained.

(2) R. Lespieau, *Bull. soc. chim.*, [4] 43, 204 (1928).

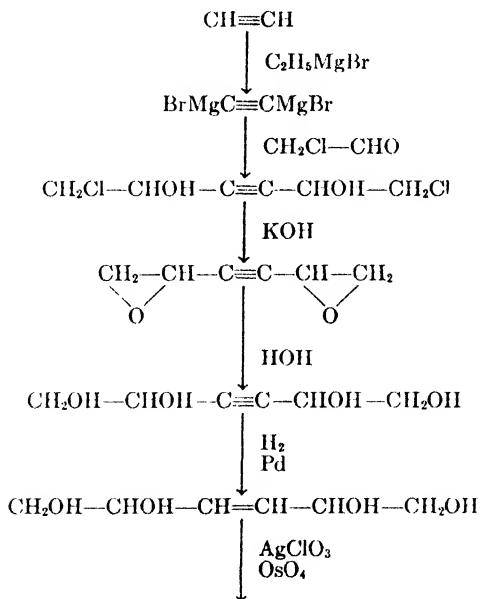
(3) M. Bourguel, *Bull. soc. chim.*, [4] 45, 1067 (1929).

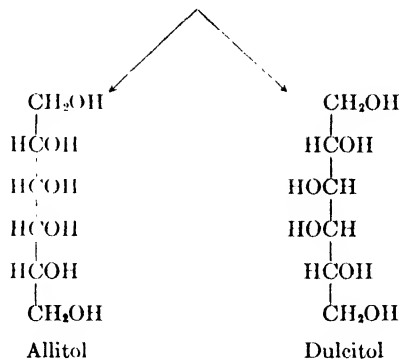
It may be well to state at this point that the reagents used in any of the syntheses which follow are sufficiently mild to preclude the possibility of rearrangement of the hydrogen atoms on the carbon chain. Thus in the compounds which lead to allitol, those hydrogen atoms which are attached to secondary carbon atoms all lie on the same side of the carbon chain, just as they do in allitol.

Hence, the precursors of allitol are the meso form of divinylglycol in the first of the above syntheses, and the meso form of the hexynetetrol and hexenetetrol in the second method of synthesis.



It is to be noted that reduction of the acetylenic compound to the olefin with Bourguel's catalyst<sup>3</sup> (palladium on starch) leads exclusively to the *cis* isomer.





## 2. Experimental Procedures

*a. Divinylglycol.* Divinylglycol was prepared according to Griner's directions.<sup>1</sup> The yield was about 45% of the theoretical.\* The product was shown to be a mixture of meso and *d,l* isomers in the following manner. Using the procedure of van Romburgh,<sup>4</sup> four atoms of bromine were added to the product. Two tetrabromides were formed, one melting at 174°, the other at 96°. The first of these, on debromination with zinc dust in alcohol, yielded a divinylglycol melting at 18°. Its isomer, m. p. 14°, has since been prepared.<sup>4b</sup> Young and coworkers<sup>5</sup> later showed, by reduction to the diethylglycols, that the divinylglycol of Griner was an equal mixture of meso and *d,l* forms.

An amount of 16 g. of the glycol (m. p. 18°) dissolved in 250 ml. of water was oxidized with 18 g. of silver chlorate and 0.3 g. of osmic acid. The reaction mixture yielded 3 g. of allitol and no *D,L*-mannitol. We may therefore assign the meso configuration to the divinylglycol melting at 18°, since on hydroxylation it yielded allitol, but not *D,L*-mannitol.

It may be mentioned at this point that the synthesis of hexitols from Griner's liquid is more easily accomplished if the mixture of isomeric divinylglycols is oxidized directly, since their separation is more difficult than the separation of the resulting hexitols.

*b. Oxidation of Divinylglycol with Silver Chlorate and Osmic Acid.*<sup>6</sup> In a typical experiment 100 g. of divinylglycol, 118 g. of silver chlorate and 1 g. of osmic acid were dissolved in 6 liters of water. The reaction mixture was kept at room temperature for several days. It was apparently essential that the reaction be carried out rather slowly.

The reaction is complete when the liquid becomes black, due to reduction of the osmic acid. Silver chloride was then removed by filtration and the

\* Note. The zinc (0.2 mm. in thickness) was converted to a zinc-copper couple by adding to it a dilute aqueous solution of copper sulfate. We took care to maintain the reaction mixture below 15° when the acetic acid was added.

(4a) P. van Romburgh and W. van Hasselt, *Proc. Acad. Sci. Amsterdam*, **35**, 40 (1932); (b) J. Wiemann, *Ann. chim.*, [11] **5**, 308 (1936).

(5) W. G. Young, S. J. Cristol and F. T. Weiss, *J. Am. Chem. Soc.*, **65**, 1245 (1943).

(6a) R. Lespieau and J. Wiemann, *Bull. soc. chim.*, [4] **53**, 1107 (1933); (b) J. Wiemann, *Ann. chim.*, [11] **5**, 267-336 (1936); (c) R. Lespieau and J. Wiemann, *Compt. rend.*, **194**, 1946 (1932).

filtrate was extracted with benzene to remove a small amount of unreduced osmic acid. The aqueous solution was next concentrated at less than 50° under reduced pressure. The black sirup thus obtained was treated with four volumes of 95% alcohol and allowed to stand until crystallization began; this usually occurred within twenty-four hours. Once initiated, the crystallization could be hastened by shaking and scratching the walls of the flask. Crystallization appeared to be complete after two to three days. The crystals were collected and there was obtained in this manner 10 g. of D,L-mannitol.

The mother liquor was then treated with a small amount of ether, whereupon it separated into two layers. The lower layer consisted of a black sirup, which, when treated with absolute alcohol very slowly deposited some crystals, 1 g., consisting largely of D,L-mannitol. The upper layer was treated with a large volume of ether, which caused the precipitation of a considerable quantity of a brown sirup. Crystallization began almost immediately, especially when the sirup was treated with absolute alcohol. The crystals were collected and there was obtained in this way 12 g. of crude allitol. After solution in water and precipitation with alcohol, the crystals melted at 140°, but still contained a small amount of impurity. After several recrystallizations they melted at 149°.

Upon standing, the mother liquor deposited more allitol—some 3 g. after a period of two months. No crystalline material was obtained from the ethereal solution. We thus obtained from 100 g. of divinylglycol, 11 g. of D,L-mannitol and 15 g. of allitol.

It was observed in those cases in which the oxidation was arrested too soon that an appreciable amount of silver chloride was deposited when the solution was concentrated. Furthermore, the mother liquor, when treated with phenylhydrazine in acetic acid, deposited some yellow crystalline D,L-mannose phenylhydrazone, m. p. 195–200° (Maquenne block). It is apparent that too vigorous oxidation results in the formation of hexoses.

*c. Identification of D,L-Mannitol and Allitol.* D,L-Mannitol was recrystallized from alcohol, and identified by melting point, 168°, and elementary analysis. It was readily soluble in water but difficultly so in alcohol. Treated with benzaldehyde in the presence of hydrochloric acid it yielded a tribenzylidene derivative melting at 192°. The racemic mannitol prepared by Fischer also melted at 168° and gave a tribenzylidene derivative melting at 192°.

Allitol was characterized by its melting point, 149–150°, elementary analysis, and by the formation of a dibenzylidene derivative, which, after recrystallization from alcohol, melted at 249–250° on the Maquenne block. Later, Steiger and Reichstein<sup>7</sup> repeated this synthesis of allitol and demonstrated the identity of the product with that obtained by the reduction of D-allose.

*d. Divinylacetylene Dioxide.*<sup>2</sup> The starting material for the synthesis of dulcitol and allitol<sup>8</sup> is the Grignard reagent derived from acetylene. In a round-bottomed flask fitted with a stirrer, reflux condenser, and gas inlet tube, there was placed 24 g. of magnesium and a large excess of ether. Some ethyl bromide was then added and when the magnesium had reacted completely, a slow stream of acetylene was led into the reaction mixture. The acetylene had previously been freed from acetone by slowly passing the gas through water, after which the acetylene was dried over calcium chloride.

(7) Marguerite Steiger and T. Reichstein, *Helv. Chim. Acta*, **19**, 188 (1936).

(8) R. Lespieau, *Bull. soc. chim.*, [5] **1**, 1374 (1934).

After several hours the reaction mixture separated into two layers. The introduction of acetylene was stopped one to two hours after the volume of the lower layer no longer appeared to change.

An ethereal solution of the aldehyde with which the acetylenic Grignard reagent was to react was then added dropwise with stirring. A little less than one mole of the aldehyde was used per mole of the Grignard reagent if only one of the magnesium atoms was to be replaced, it being impossible, however, to avoid some replacement of both magnesium atoms. If, as in this case, the latter reaction was desired, two moles of the aldehyde were added. Stirring was continued for about an hour after the addition of the aldehyde was complete; dilute hydrochloric acid was then added, after which the ethereal layer was separated and the ether removed by evaporation.

Chloroacetaldehyde was prepared by heating its crystalline polymer and collecting the distillate in ether. The ethereal solution was at once reacted with the acetylenic Grignard reagent according to the procedure described above. After removal of the ether, the reaction mixture was concentrated as far as possible with a water pump. The residue at this point contained *meso*-divinylacetylene dichlorohydrin,  $\text{CH}_2\text{Cl}-\text{CHOH}-\text{C}\equiv\text{C}-\text{CHOH}-\text{CH}_2\text{Cl}$ , and probably the *d,l* isomer. When distillation was attempted by heating the reaction mixture strongly, it decomposed in an explosive manner.

In order to convert the dichlorohydrin into the corresponding diepoxy compound it was redissolved in ether and about one and one-half times the theoretical amount of dry pulverized potassium hydroxide was added slowly with vigorous agitation. The resulting solution, after filtration and removal of excess solvent, was distilled through an efficient column and yielded *meso*-divinylacetylene dioxide,  $\text{CH}_2-\text{CH}=\text{C}=\text{CH}-\text{CH}_2$ , b. p.  $98-99^\circ_{20\text{ mm}}$ .



$d_{25} 1.1189$ ,  $n_D^{25} 1.4871$ . The compound showed a strong exaltation of the molecular refraction, a normal phenomenon.

*e. 1,2,5,6-Tetrahydroxyhexyne-3.* Divinylacetylene dioxide was hydrolyzed to the corresponding tetrahydroxyhexyne,  $\text{CH}_2\text{OH}-\text{CHOH}-\text{C}\equiv\text{C}-\text{CHOH}-\text{CH}_2\text{OH}$ , by heating an aqueous solution of the dioxide for some time at  $100^\circ$  in a sealed tube. The addition of a few drops of sulfuric acid to the solution caused the reaction to become too vigorous.

Evaporation of the water gave a partially crystalline mass which was dissolved in methanol. Evaporation of the methanol yielded a thick paste, from which more solvent was removed by pressing it between filter paper. After several repetitions of this procedure the product sintered somewhat about  $100^\circ$ , but had no sharp melting point. It was then recrystallized by evaporation of solvent from its solution, first in alcohol and then in acetic acid. The crystalline material thus obtained melted at  $113-114.5^\circ$  and gave good analytical values, but a purer product resulted from the treatment of a methanolic solution of this material with enough ether to form a second layer. A reddish flocculent precipitate was separated and after evaporation of the alcohol, crystals melting at  $116-117.5^\circ$  were obtained.

*f. 1,2,5,6-Tetrahydroxyhexene-3.* The hexynetetrol obtained above was reduced to the corresponding hexenetetrol by hydrogenation over Bourguel's catalyst.<sup>3</sup> In a typical experiment the hexynetetrol, alone, or in aqueous or dilute alcoholic solution, was placed in a filter flask of at least three times the volume of the solution to be reduced, together with perhaps 1 ml. of Bourguel's



catalyst (0.01 g. palladium). Hydrogen was introduced into the flask through the side arm which was connected to a 5-liter bottle filled with the gas. A second bottle connected with the first and filled with water served to maintain a slight positive pressure on the hydrogen. After the filter flask had been flushed with hydrogen it was stoppered and placed on a shaking machine.

The absorption of hydrogen occurred smoothly at a rate which could be followed by the rise of water in the bottle containing hydrogen. When this rate suddenly decreased it was known that two atoms of hydrogen had been added to the triple bond. The hexenetetrol, which was formed in excellent yield, melted at 80–82°.

*g. 1,2,5,6-Tetraacetoxyhexene-3.* The hexenetetrol obtained above was acetylated with acetic anhydride in the presence of a small amount of potassium acetate. The tetraacetate,  $\text{CH}_2\text{OAc}-\text{CHOAc}-\text{CH}=\text{CH}-\text{CHOAc}-\text{CH}_2\text{OAc}$ , had the following constants: b. p.<sub>12 mm.</sub> 170°,  $d_{20}$  1.186,  $n_D^{20}$  1.462.

*h. Allitol and Dulcitol.* Oxidation of 1,2,5,6-tetrahydroxyhexene-3 with silver chlorate and osmic acid as described above yielded the corresponding hexitol. In a typical experiment, 4 g. of the hexenetetrol was oxidized with 1.73 g. of silver chlorate and 0.055 g. of osmic acid, yielding 0.55 g. of crude allitol. The product began to sinter at 142° and was completely melted at 148°, except for a trace of crystalline material which melted only above 160°. The impurity could be eliminated by fractional fusion but there was too little material present to permit further investigation. From later work it became apparent that the impurity in this case was dulcitol.

Oxidation of 1,2,5,6-tetraacetoxyhexene-3 led to the formation of dulcitol tetraacetate. In a typical experiment 22 g. of the tetraacetate was oxidized with 4.4 g. of silver chlorate and 0.1 g. of osmic acid and yielded a sirup which did not crystallize. The sirup was acetylated again and yielded the corresponding hexitol hexaacetate. The crystals which deposited first were collected and recrystallized from methanol, in which they are difficultly soluble in the cold. The product melted at 166–166.5° and analyzed correctly for a hexitol hexaacetate. Now there was found in the collections of the École Normale Supérieure an old bottle containing dulcitol hexaacetate, m. p. 167–168°. A mixture of the new and the old hexaacetates was found to melt at 166.5–167.5°; the identity of the two compounds was thus beyond doubt.

The dulcitol hexaacetate mother liquor yielded a small quantity of crystalline material, m. p. 50–59°, whose nature has not been determined. Its melting point is near that of allitol hexaacetate (m. p. 61–62°).<sup>6b</sup>

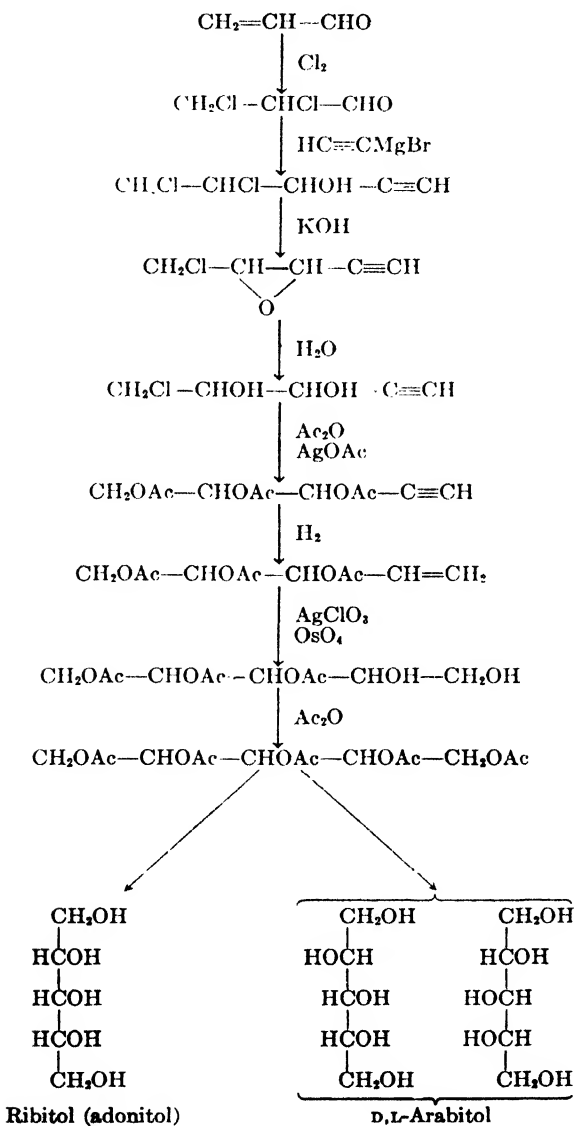
### III. SYNTHESIS OF PENTITOLS FROM UNSATURATED INTERMEDIATES

#### 1. Methods of Preparation

Very little work on the synthesis of pentitols had been carried out before 1928. Those pentitols which had been synthesized were made by the reduction of pentoses. Several pentitol intermediates had been synthesized<sup>9</sup> and it seemed desirable to attempt to complete the synthesis of pentitols from the unsaturated intermediates, using the methods which had been successfully employed in the synthesis of hexitols.

(9) R. Lespieau, *Bull. soc. chim.*, [4] 43, 657 (1928).

The desired intermediates, a pentadienol or a pentenetriol were unknown at the time, but it occurred to me that their synthesis might be accomplished by the partial reduction of a pentenynol or a pentynetriol, the latter being obtained from the reaction of one molecule of acrolein or acrolein dichloride with the Grignard reagent derived from acetylene. The reactions which have been carried out in accordance with this scheme are shown in the accompanying flow sheet.



## 2. Experimental Procedures<sup>9</sup>

*a. 3-Hydroxy-4,5-dichloropentyne-1.* Acrolein dichloride was prepared by saturating acrolein at 0° with gaseous chlorine and distilling the product. The Grignard reagent derived from acetylene was prepared as described previously (page 112). In a typical preparation using 24 g. of magnesium, 50 g. of acrolein dichloride was added slowly and with vigorous agitation to the acetylenic Grignard reagent dissolved in a large volume of ether. After standing for twenty-four hours, the reaction mixture was made slightly acid and extracted with ether. Following removal of the solvent the residue was steam distilled. The product, 3-hydroxy-4,5-dichloropentyne-1,  $\text{CH}_2\text{Cl}-\text{CHCl}-\text{CHOH}-\text{C}\equiv\text{CH}$ , was obtained from the distillate in an amount equal to 7-13% of the weight of the acrolein dichloride used. It had the following constants: b. p. 90-91°<sub>12 mm.</sub>,  $d_{23}$  1.306,  $n_D^{23}$  1.500.

*b. 3,4-Epoxy-5-chloropentyne-1.* The dichlorohydroxypentyne obtained above was converted to the corresponding epichlorohydrin,  $\text{CH}_2\text{Cl}-\text{CH}-\text{CH}-\text{C}\equiv\text{CH}$ , in 85% yield by vigorous agitation with the theoretical

amount of 10% aqueous potassium hydroxide for four hours, followed by extraction with ether. However, in one experiment using 100 g. of the dichlorohydroxypentyne, scarcely any product was obtained other than a yellow chlorine-free resin. It would seem advisable not to work with too large an amount of material at one time.

The epichlorohydrin is a colorless liquid with an odor much like that of ordinary epichlorohydrin and the following constants: b. p. 152°, b. p.<sub>20 mm.</sub> 58-60°,  $d_{23}$  1.159,  $n_D^{23}$  1.472. The usual exaltation of the molecular refraction was encountered.

*c. 3,4-Dihydroxy-5-chloropentyne-1.* The preceding epichlorohydrin was converted to the monochlorohydrin,  $\text{CH}_2\text{Cl}-\text{CHOH}-\text{CHOH}-\text{C}\equiv\text{CH}$ , by hydration. In a typical experiment 12.5 g. of the epichlorohydrin and 25 g. of water were heated in a sealed tube at 100° for thirty hours. After removal of the water by heating at 30° under reduced pressure, the residue yielded 2 g. of a liquid boiling up to 133° at 16 mm., 9.5 g. boiling at 133-136°<sub>16 mm.</sub> and traces of material boiling at 140-145°<sub>16 mm.</sub>. After a second distillation the monochlorohydrin was obtained as a liquid boiling at 131-132° at 16 mm.,  $d_{16}$  1.289,  $n_D^{16}$  1.504. The liquid solidified on standing and the air-dried crystals, no longer deliquescent, were recrystallized by evaporation of their benzene solution. The very fine white needles first deposited matted down rapidly. They melted at 41-42°.

*d. 3,4,5-Triacetoxypentyne-1.* The monochlorohydrin obtained above was converted to the corresponding triacetate,  $\text{CH}_2\text{OAc}-\text{CHOAc}-\text{CHOAc}-\text{C}\equiv\text{CH}$ , by refluxing it for one hour with one and one-half moles of acetic anhydride and one mole of silver acetate. Ether was added to the cooled reaction mixture, which was then filtered and distilled. The product had the following constants: b. p. 149.5-150.5°<sub>9 mm.</sub>,  $d_{24.5}$  1.1436,  $n_D^{24.5}$  1.445.

*e. 3,4,5-Triacetoxypentene-1.* The pentyne triacetate obtained above was hydrogenated with Bourguel's catalyst<sup>3</sup> to the pentene triacetate,  $\text{CH}_2\text{OAc}-\text{CHOAc}-\text{CHOAc}-\text{CH}=\text{CH}_2$ , b. p. 148-149°<sub>14 mm.</sub>,  $d_{18.5}$  1.1178,  $n_D^{18.5}$  1.4418.

*f. 3,4,5-Triacetoxypentenediol-1,2.* The preceding triacetoxypentyne was converted to a mixture of two isomeric pentitol triacetates,  $\text{CH}_2\text{OAc}-$

$\text{CHOAc}-\text{CHOAc}-\text{CHOH}-\text{CH}_2\text{OH}$ , by the action of silver chlorate and osmic acid.

*g. D,L-Arabitol.*<sup>10</sup> The pentitol triacetate mixture obtained above was heated with a slight excess of acetic anhydride to form the corresponding pentaacetate,  $\text{CH}_2\text{OAc}-\text{CHOAc}-\text{CHOAc}-\text{CHOAc}-\text{CH}_2\text{OAc}$ . After removal of excess acetic anhydride and acetic acid by concentration under reduced pressure, the residue consisted of a white crystalline solid and an approximately equal amount of liquid. After recrystallization from methanol the solid melted at  $96-96.5^\circ$  and was characterized as a pentitol pentaacetate by elementary analysis, saponification equivalent, and molecular weight determination. The crystalline solid was further identified as *D,L*-arabitol pentaacetate by its melting point which was the same as that of *D,L*-arabitol pentaacetate prepared by Asahina and Yanagita.<sup>11</sup> In 1934 these workers discovered that certain lichens contained *D*-arabitol, and in order to identify this substance they mixed it with an equal weight of *L*-arabitol. The *D,L*-arabitol thus formed had the correct melting point for the racemate and the pentaacetate formed on acetylation melted at  $95^\circ$ .

The *D,L*-arabitol pentaacetate was hydrolyzed by refluxing it for three hours with methanol containing an excess (7 moles) of hydrogen chloride. After concentration to one-half its volume the solution crystallized spontaneously. By collecting the product before crystallization was too far advanced and washing it with a little fresh methanol, crystals melting at  $105-106^\circ$  were obtained. This is the melting point of *D,L*-arabitol as first recorded by Ruff.<sup>12</sup>

*h. Ribitol.*<sup>13</sup> The sirup obtained along with the crystalline *D,L*-arabitol pentaacetate was slightly brownish in color and had not crystallized at the end of a year's time. It was then distilled under high vacuum. There was obtained in this way about 20 g. of a colorless liquid having the following constants: *b. p.*  $110^{+0.006}_{-0.006}$  mm.;  $d_{25}$  1.2143;  $n_D^{25}$  1.444; molecular refraction, 79.19 (calculated for  $\text{C}_5\text{H}_7(\text{OAc})_6$ , 79.64).

The liquid began to crystallize on standing, but after several months' time crystallization appeared to stop and the crystalline material (2 g.) was removed. The latter proved to be identical with the *D,L*-arabitol pentaacetate obtained above, the total yield of which was approximately equal to that of the liquid which did not crystallize.

This liquid was hydrolyzed under conditions similar to those employed with *D,L*-arabitol pentaacetate and yielded a sirup which crystallized spontaneously on standing, or immediately if seeded with ribitol. The crude product melted at  $96-104^\circ$ ; upon recrystallization from ethanol the melting point and mixed melting point with an authentic sample of ribitol was  $101.5-102^\circ$ .

*i. Other Pentitol Pentaacetates.* 3-Hydroxypentene-1-yne-4,  $\text{CH}_2=\text{CH}-\text{CHOH}-\text{C}\equiv\text{CH}$ , was obtained in a yield of 11 g. from the reaction of 25 g. of acrolein with the acetylenic Grignard reagent obtained from 50 g. of magnesium. Only 2 g. of the glycol,  $\text{CH}_2=\text{CH}-\text{CHOH}-\text{C}\equiv\text{C}-\text{CHOH}-\text{CH}=\text{CH}_2$ , was

(10) R. Lespiau, *Compt. rend.*, **203**, 145 (1936).

(11) Y. Asahina and M. Yanagita, *Ber.*, **67B**, 799 (1934).

(12) O. Ruff, *Ber.*, **32**, 556 (1899).

(13) R. Lespiau, *Compt. rend.*, **206**, 1773 (1938).

formed simultaneously. The pentenynol boils at 128.5–129.5° and requires certain precautions in its isolation.<sup>14</sup>

Hydrogenation of the pentenynol with Bourguel's catalyst<sup>1</sup> gives the corresponding divinylcarbinol,  $\text{CH}_2=\text{CH}-\text{CHOH}-\text{CH}=\text{CH}_2$ , b. p. 114.5–116°,  $d_{19}$  0.8648,  $n_D^{19}$  1.4452. Treatment of this divinylcarbinol with the theoretical quantity of silver chlorate and a little osmic acid yielded a sirup which has not crystallized. From the acetylation of this sirup there was obtained a small quantity of D,L-arabitol pentaacetate and a viscous liquid which has not been studied further but certainly contains one or both of the other pentitol pentaacetates.

- (14) R. Lespiau and R. Lombard, *Bull. soc. chim.*, [5] 2, 369 (1935).

# THE INTERRELATION OF CARBOHYDRATE AND FAT METABOLISM

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## I. INTRODUCTION

Although it is becoming increasingly evident with each new discovery that the metabolism of no single foodstuff proceeds in a course entirely isolated from that of the other foodstuffs, the interrelationships are nowhere so apparent as they are between the utilization of carbohydrate and that of fat. The conversion of carbohydrate to fat is a phenomenon which has been repeatedly demonstrated experimentally; the reverse change, namely, the conversion of natural fats to carbohydrate, is a disputed reaction and must certainly not occur except to a limited extent, if at all.

In addition to these interconversions, the metabolism of fat and the metabolism of carbohydrate are inseparably related. This fact is most clearly demonstrated by the appearance of such abnormal products of fat oxidation as the so-called "ketone bodies" in the blood and urine whenever the supply of carbohydrate is deficient or in cases where the organism is unable to metabolize this foodstuff. Whether ketonuria results because the metabolism of fat must occur concomitantly with that of D-glucose (ketolysis), or whether the presence of D-glucose prevents any fat breakdown because it is preferentially oxidized (antiketogenesis) is still a moot question.

It is also believed by some workers that the ability to utilize certain fats may be related to the type of carbohydrate that is present in the diet. In the present review, the evidence concerning these various relationships will be presented.

## II. THE TRANSFORMATION OF CARBOHYDRATE TO FAT

### 1. *Experimental Proof*<sup>1</sup>

It has long been recognized that foodstuffs other than lipids can be a source of body fat. The wide usage of corn as a fattening agent for hogs

(1) An excellent review of various interconversions of the foodstuffs, with an extensive bibliography, is given by D. Rapport, *Physiol. Revs.*, 10, 349 (1930).

is certainly not dependent on the relatively low content of oil in this cereal; there is no doubt that the lard arises largely from the corn starch, which makes up over 70% of the grain. The experimental proof of such a transformation was first adduced by Meissl and Strohmer<sup>2</sup> in 1883 by the use of balance experiments. These authors demonstrated that a considerable proportion of the carbon of the ingested rice could not be accounted for in the excreta (urine, feces, and expired air). After subtraction of the small amount which could have been retained as protein, a large balance still remained which far exceeded the carbon present in the total carbohydrate reserves of the hog. The authors concluded that the missing carbon could be stored in the body only in the form of fat. Balance experiments also have demonstrated the conversion of carbohydrate into fat in the goose<sup>3</sup> and in the dog.<sup>4</sup>

Another indirect method which has given further evidence for the transformation of carbohydrate to fat is based on the fact that elevated levels of the Respiratory Quotient (R. Q.; volume of respiratory CO<sub>2</sub>/volume of respiratory O<sub>2</sub>) have been observed during periods when fat synthesis from carbohydrate is believed to be progressing at a rapid rate.<sup>5</sup> We have much evidence that the relative carbon dioxide production and oxygen requirement afford an index of the composition of the food which is being metabolized. When an oxygen-rich foodstuff, such as carbohydrate, is being converted to an oxygen-poor one, such as fat, the extra oxygen is available for metabolic purposes and accordingly less oxygen needs to be obtained from the respiratory gases. Inasmuch as the oxidative processes that are necessary for life are also maintained, carbon dioxide from these completely oxidized metabolites continues to be formed and to be excreted at an undiminished rate. As a consequence, R. Q.'s exceeding 1.0, which is the highest for the metabolism of any single foodstuff (carbohydrate), may be expected. A further augmentation, additional to that from the extra oxygen left over from the carbohydrate molecules, may also result from the extra carbon dioxide produced when carbohydrate is converted to fat if Bleibtreu's equation<sup>6</sup> for the conversion of D-glucose to palmitic acid is correct. This is as follows:



(2) E. Meissl and F. Strohmer, *Sitzber. Akad. Wiss. Wien. Math.-naturw. Klasse, Abt III*, **87**, 205 (1883).

(3) K. B. Lehmann and E. Voit, *Z. Biol.*, **42**, 644 (1901).

(4) M. Rubner, quoted by G. Lusk on p. 395 of the book referred to in note 10.

(5) For a complete discussion of the respiratory quotient and its metabolic implications, see H. B. Richardson, *Physiol. Revs.*, **9**, 61 (1929).

(6) M. Bleibtreu, *Arch. ges. Physiol. (Pflügers)*, **85**, 345 (1901); cf. E. Meissl, *Z. Biol.*, **22**, 142 (1886).



A maximum R. Q. of 8.0 would therefore obtain if fat synthesis from carbohydrate were alone taking place. However, in the myriad of changes continually occurring, such a reaction can scarcely play a major role. Considerably augmented levels of R. Q. have, however, been observed experimentally in animals which have received excessive quantities of carbohydrate. Bleibtreu<sup>6</sup> first observed a R. Q. of 1.33 in a goose stuffed with carbohydrate although the same animal when fasted had a R. Q. of 0.73. Pembrey<sup>7</sup> reported a ratio of 1.39 in a marmot previous to hibernation, while Grafe<sup>8</sup> found a non-protein R. Q. of 1.31 for dogs given 300% of their daily caloric requirement as carbohydrate. The most extended observations of these phenomena are reported by Wierzechowski and Ling<sup>9</sup> in Lusk's laboratory with a young hog. After the ingestion of 700 g. of starch, a R. Q. of approximately 1.40 was maintained for over twenty hours with a maximum value of 1.58. It was calculated that as much as 7.1 g. of fat were manufactured per hour; also a total of 125 g. of fat was produced per day with an average hourly production of 5.2. Under these unusual circumstances only 27% of the metabolized starch was required to satisfy the basal metabolism although 13% was expended for specific dynamic action. More than half of the energy of the starch (56%) was calculated as being converted to that of fat. The change of carbohydrate to fat apparently can take place both in the liver and in the extrahepatic tissues.<sup>9a</sup> It has also been shown that adipose tissue itself possesses the ability of fat synthesis from carbohydrate.<sup>9b, 9c</sup>

## 2. Mechanism of Transformation

We have no experimental evidence on the exact intermediary changes which occur in the complex transformations necessary to convert sugar into fat. The quantities of any such intermediates that may be present in the tissues at any one time are probably so small as to make their detection a relatively hopeless task. Lusk<sup>10</sup> calculates that a 13.5 kg. hog can synthesize a maximum of 2 mg. of fat per second. If such transformations are generalized throughout the organism, this rate would involve a concentration at any instant of only 0.15  $\mu$ g. per g. of tissue.

(7) M. S. Pembrey, *J. Physiol.*, **27**, 407 (1901-02).

(8) E. Grafe, *Deut. Arch. klin. Med.*, **113**, 1 (1914).

(9) M. Wierzechowski and S. M. Ling, *J. Biol. Chem.*, **64**, 697 (1925).

(9a) J. Tepperman, J. R. Brobeck and C. N. H. Long, *Yale J. Biol. Med.*, **15**, 855 (1943).

(9b) E. Tuerkischer and E. Wertheimer, *J. Physiol.*, **100**, 385 (1941-42).

(9c) A. Mirski, *Biochem. J.*, **36**, 232 (1942).

(10) G. Lusk, "The Elements of the Science of Nutrition," W. B. Saunders Co., Philadelphia and London, 4th ed., p. 398 (1928).

Since the chief fatty acids which are synthesized from carbohydrate are  $C_{16}$  and  $C_{18}$  acids,<sup>11, 12</sup> it would seem that any intermediate would necessarily contain the number of carbon atoms that would be a common denominator of these values, namely two. Of course, it is also possible to postulate that three D-glucose molecules can be converted into a single  $C_{18}$  acid, which can then be degraded to a  $C_{16}$  acid. The conversion of stearic into palmitic acid *in vivo* has been proved by Schoenheimer and Rittenberg,<sup>13</sup> and one might assume that a further similar degradation might account for the formation of the shorter-chain fatty acids in the tissues as a result of progressive  $\beta$ -oxidations.

In an extension of the first theory, Magnus-Levy<sup>14</sup> has suggested that acetaldehyde may be the 2-carbon intermediate in question. It is postulated that a condensation occurs (similar to the aldol condensation) followed by a reduction of the hydroxyl group to form butyraldehyde. Successive condensations with new acetaldehyde molecules follow along with subsequent reductions of the  $\beta$ -hydroxyl groups. When the chain has reached the desired length, an oxidation of the aldehyde group to acid occurs, and the neutral fat may be synthesized by combination with glycerol, which can readily be formed from D-glucose. The presence in tissues of the aldehydes corresponding to palmitic and stearic acids has been demonstrated by Feulgen and coworkers.<sup>15, 16</sup> Möckel<sup>17</sup> has noted that such lipoaldehydes are present in increased amounts as components of plasmochem in tissues whenever fat synthesis or degradation occurs. Smedley<sup>17a, 17b</sup> has suggested that the synthesis of fatty acids involves an aldol condensation with pyruvic acid.

Although there is little direct evidence that acetaldehyde may serve as the important intermediate, it has been reported as a component of urine.<sup>18</sup> Such a scheme could explain the universal appearance of even-chain fatty acids in the tissues as well as does the suggestion that they originate by a series of  $\beta$ -oxidations from longer-chain acids.

An especially cogent argument for the acetaldehyde theory is the fact that considerable amounts of pyruvic acid accumulate in the organism

(11) H. E. Longenecker, *J. Biol. Chem.*, **128**, 645 (1939).

(12) H. E. Longenecker, G. Gavin and E. W. McHenry, *J. Biol. Chem.*, **134**, 693 (1940).

(13) R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, **120**, 155 (1937).

(14) A. Magnus-Levy, *Arch. ges. Physiol. (Pflügers)*, **55**, 103 (1894).

(15) R. Feulgen, J. Imhäuser and M. Behrens, *Z. physiol. Chem.*, **180**, 161 (1929).

(16) R. Feulgen and M. Behrens, *Z. physiol. Chem.*, **256**, 15 (1938).

(17) G. Möckel, *Z. physiol. Chem.*, **277**, 135 (1943).

(17a) I. Smedley, *J. Physiol.*, **45**, xxv (1912-13).

(17b) I. Smedley and E. Lubrznyska, *Biochem. J.*, **7**, 364 (1913).

(18) W. Stepp and R. Feulgen, *Z. physiol. Chem.*, **119**, 72 (1922).

as a result of the metabolism of carbohydrates, and this can readily be converted to acetaldehyde by the activity of the enzyme carboxylase.<sup>19</sup> Such a reaction requires the presence of a coenzyme, co-carboxylase, which has been found to be thiamine pyrophosphate. That a similar mechanism controls this reaction *in vivo* is indicated by the fact that pyruvic acid excretion is markedly increased by thiamine deficiency, especially when carbohydrate is ingested.<sup>20, 20a</sup> Since it has been found that thiamine is a requirement for fat formation,<sup>12, 21-23</sup> this would seem to offer presumptive evidence that the pyruvate  $\rightarrow$  acetaldehyde change is concerned with this transformation and that therefore acetaldehyde is probably an intermediate. Quackenbush, Steenbock and Platz<sup>24</sup> have suggested that not only thiamine but also other members of the B-complex are important in fat synthesis.

Additional proof of the importance of thiamine in fat formation from carbohydrate is afforded by the work of Ring.<sup>25</sup> He found that when D-glucose and thiamine were given together the specific dynamic action was twice that resulting from D-glucose alone. Inasmuch as no effect on heat production followed the administration of thiamine alone or with fat, it is considered that the extra heat produced when thiamine and carbohydrate are given together is to be ascribed to the waste energy set free in the change of carbohydrate to fat.

Until recently there has been no evidence that acetic acid plays any role in the formation of long-chain fatty acids in the animal body.<sup>26</sup> It has now been reported that considerable amounts of the carbon isotope C<sup>13</sup> incorporated in the carboxyl group of acetic acid do find their way not only into the carboxyl group of the new fatty acid but also into the interior of the molecule. This leads these authors<sup>27</sup> to the conclusion that the long-chain fatty acids may be synthesized by condensations of successive C<sub>2</sub> units. In addition, it was proved that the methyl group is utilized in such a synthesis because an accumulation of the deuterium from the deuterio-methyl group of the acetic acid occurred in the body fat. However, no evidence that long-chain fatty acids could be synthesized

(19) C. Neuberger and L. Karczag, *Biochem. Z.*, **36**, 68 (1911).

(20) H. A. Harper and H. J. Deuel, Jr., *J. Biol. Chem.*, **137**, 233 (1941).

(20a) M. E. Shils, H. G. Day and E. V. McCollum, *J. Biol. Chem.*, **139**, 145 (1941).

(21) D. V. Whipple and C. F. Church, *J. Biol. Chem.*, **114**, cvii (1936).

(22) E. W. McHenry, *Science*, **86**, 200 (1937).

(23) E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **125**, 653 (1938).

(24) F. W. Quackenbush, H. Steenbock and B. R. Platz, *J. Biol. Chem.*, **145**, 163 (1942).

(25) G. C. Ring, *Am. J. Physiol.*, **138**, 488 (1942-43).

(26) K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **145**, 625 (1942).

(27) D. Rittenberg and K. Bloch, *J. Biol. Chem.*, **154**, 311 (1944); **60**, 417 (1945).

from butyric acid was obtained by Morehouse<sup>28</sup> when deuterio-tributyrim was fed to rats.

### 3. *The Nature of the Fat Formed from Carbohydrate*

A larger proportion of saturated to unsaturated triglycerides is present in the depot fats of rats which have received a high carbohydrate, fat-free diet than in those on an ordinary mixed diet where the dietary fat, after slight alterations, may contribute to the body lipid stores. Anderson and Mendel<sup>29</sup> have demonstrated that when "soft" body fat has been developed by certain types of diets, it may be hardened by changing the animals to a high carbohydrate diet. The fat laid down in the latter case has a considerably lower iodine number than that produced after a diet containing soybean oil; the mean values were 118 to 101 before the carbohydrate diet and 57 to 50 following it.<sup>30</sup> Similar decreases in the iodine number of fat deposited after the feeding of carbohydrate have been reported in hogs.<sup>31</sup> There is, however, a considerable amount of a mono-unsaturated acid (hexadecenoic acid or palmitoleic acid) laid down in rats on a high carbohydrate diet. Longenecker<sup>11</sup> found that although only about 5% of this acid was present in the fat of rats on a normal diet, it was increased to as high as 13% after a high carbohydrate regimen and approximately 16% on a diet almost exclusively protein. In addition, definite but less striking increases in the amount of palmitic acid were noted.

## III. THE TRANSFORMATION OF FAT TO CARBOHYDRATE

In contrast with the extensive experimental evidence for the transformation of carbohydrate to fat, there is no indisputable and clear-cut proof in the higher animal of the reverse change of fatty acid to carbohydrate. Few fields in biochemistry afford so large a mass of polemical reports and so little decisive evidence as does the record concerning this subject. The acceptance or rejection of the theory that fat can be transformed to carbohydrate has largely been related to the views of the investigator as to the cause of diabetes. Those who accept the "overproduction" theory hold that the hyperglycemia and glycosuria of diabetes mellitus are the result of an increased production of sugar from fat, while those who advocate the "non-oxidation" theory believe that

(28) M. G. Morehouse, *J. Biol. Chem.*, **155**, 33 (1944).

(29) W. E. Anderson and L. B. Mendel, *J. Biol. Chem.*, **76**, 729 (1928).

(30) L. L. Reed, F. Yamaguchi, W. E. Anderson and L. B. Mendel, *J. Biol. Chem.*, **87**, 147 (1930).

(31) N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, **69**, 219 (1926).

the sugar accumulates because of an impaired utilization of carbohydrate. Macleod<sup>32</sup> has recently been one of the strongest advocates of the "overproduction" theory while Lusk<sup>33</sup> has vigorously denied the possibility of the change. There have been a number of reviews on this subject,<sup>1, 5, 34-39a</sup> most of which have supported Lusk.

### 1. Transformation of Glycerol to D-Glucose<sup>39b</sup>

There has been unanimity of opinion that glycerol is convertible to D-glucose. This alcohol was early reported to lower the ketonuria in diabetic patients<sup>40-42</sup> as well as to be an effective agent in relieving hypoglycemia resulting from overdosage of insulin.<sup>43</sup> Cremer<sup>44</sup> found about 40% of the ingested glycerol was accounted for as "extra glucose" in the urine of the phlorizinized dog, and Luthje<sup>45</sup> reported similar results on depancreatized dogs. As a consequence, this value was for a time used for the calculation of the antiketogenic value of glycerol in the diets of diabetic patients.<sup>46, 47</sup> However, more recent experiments of Chambers and Deuel<sup>48</sup> have indicated that a complete conversion to D-glucose can take place in phlorizinized dogs. They suggest that lower values found earlier may possibly be ascribable to incomplete absorption. Further confirmation of the D-glucose-forming activity of glycerol is the demonstration that it possesses equal activity with D-glucose as a glyco-genic agent in rats.<sup>49, 50</sup> Also, it has been shown to be an effective agent

(32) J. J. R. Macleod, "The Fuel of Life," Princeton Univ. Press (1928).

(33) G. Lusk, ref. 10, pp. 639-643.

(34) C. F. Cori, *Physiol. Revs.*, **11**, 143 (1931).

(35) T. M. Carpenter, *J. Nutrition*, **4**, 281 (1931).

(36) H. C. Geelmuyden, *Ergeb. Physiol.*, **31**, 1 (1931).

(37) M. Dann, *Yale J. Biol. Med.*, **5**, 359 (1932-33).

(38) H. H. Mitchell, *J. Nutrition*, **6**, 473 (1933).

(39) S. Soskin, *Endocrinology*, **26**, 297 (1940).

(39a) W. C. Stadie, *Physiol. Revs.*, **25**, 395 (1945).

(39b) See C. J. Carr and J. C. Krantz, Jr., *Advances in Carbohydrate Chem.*, **1**, 177 (1945).

(40) F. Hirschfeld, *Z. klin. Med.*, **28**, 176 (1895).

(41) G. Satta, *Beitr. chem. Physiol. Pathol.*, **6**, 376 (1905).

(42) R. M. Lang, *Biochem. J.*, **9**, 456 (1915).

(43) C. Voegtlin, E. R. Dunn and J. W. Thompson, *Am. J. Physiol.*, **71**, 574 (1924-25).

(44) M. Cremer, *Munch. med. Wochschr.*, **49**, 944 (1902).

(45) H. Luthje, *Deut. Arch. klin. Med.*, **80**, 98 (1904).

(46) W. S. McCann, R. R. Hannon, W. A. Perlzweig and E. H. Thompkins, *Arch Internal Med.*, **32**, 226 (1923).

(47) H. M. Thomas, Jr., *Bull. Johns Hopkins Hosp.*, **35**, 201 (1924).

(48) W. H. Chambers and H. J. Deuel, Jr., *J. Biol. Chem.*, **65**, 21 (1925).

(49) I. Shapiro, *J. Biol. Chem.*, **108**, 373 (1935).

(50) L. F. Catron and H. B. Lewis, *J. Biol. Chem.*, **84**, 553 (1929).

in lowering exogenous ketonuria produced by the administration of sodium acetoacetate to fasting rats.<sup>49</sup>

However, glycogenesis has not been conclusively demonstrated for the glycerol moiety of the triglyceride when it is fed as a component of a natural fat.<sup>51, 52</sup> This is readily understandable since the quantity that it is possible to administer under such conditions (only 10% of the weight of the fat) is too small to give definite increases in liver glycogen. Moreover, the slow rate of absorption and metabolism of the fat would make any glycerol that might be available at any one period quite small. In addition, it is probable that a large part of such ingested fat may not be immediately available for oxidation but may be stored as the triglyceride; the glycerol would in such a case obviously not be available for glycogenesis. There has also been a suggestion that the glycerol moiety may be oxidized simultaneously with one of the fatty acid molecules, in which case it would exert a ketolytic effect without actually undergoing a change to D-glucose or glycogen.

When the triglycerides which cannot be stored as neutral fat (such as triacetin, tributyrin, tricaproin, or tricaprylin<sup>53-55</sup>) are fed, glycogen formation can be demonstrated.<sup>51, 52</sup> Inasmuch as the short-chain fatty acid components of these fats are incapable of being transformed to glycogen, the origin of this polysaccharide must be traced to the glycerol in the triglyceride fed. If resynthesis to the neutral fat does not occur after absorption, the glycerol would remain free to serve as a source of the glycogen. The rapid digestion and absorption of triacetin and tributyrin,<sup>56</sup> which far exceeds that of the natural fats, would aid in the rapid mobilization of the glycerol for glycogenesis. It is possible that the small amount of liver glycogen occurring in the liver of the rats previously fasted for twenty-four hours and then fed exclusively on butterfat for three to eight days may be traced to the glycerol of the triglycerides (chiefly tributyrin) which cannot be deposited in the animal body.<sup>57, 58</sup>

(51) H. J. Deuel, Jr., J. S. Butts, L. F. Hallman and C. H. Cutler, *J. Biol. Chem.*, **112**, 15 (1935-36).

(52) H. J. Deuel, Jr., J. S. Butts, H. Blunden, C. H. Cutler and L. Knott, *J. Biol. Chem.*, **117**, 119 (1937).

(53) H. C. Eckstein, *J. Biol. Chem.*, **81**, 613 (1929); **84**, 353 (1929).

(54) M. Powell, *J. Biol. Chem.*, **89**, 547 (1930).

(55) M. Powell, *J. Biol. Chem.*, **95**, 43 (1932).

(56) H. J. Deuel, Jr. and L. Hallman, *J. Nutrition*, **20**, 227 (1940).

(57) D. E. Gregg, *J. Nutrition*, **4**, 385 (1931).

(58) C. L. Gemmill and E. G. Holmes, *Biochem. J.*, **29**, 338 (1935).

## 2. Transformation of Fatty Acids with Odd-numbered Carbon Chains to Carbohydrate

The end product of the intermediary metabolism of fatty acids with an uneven number of carbons differs sharply from those formed after the breakdown of the even-chain fatty acids. Whereas glycogen is produced from the fatty acids having an uneven number of carbon atoms, those fatty acids with an even number of carbon atoms have no glycolytic activity, but, with the possible exception of acetic acid, they are all ketogenic and possess no ketolytic activity.

The simplest representative of the odd-numbered group (other than formic acid, which is atypical) is propionic acid. This substance was shown to be 100% convertible to D-glucose in the phlorizinized dog.<sup>59</sup> Further proof of its transformability to carbohydrate is afforded by the demonstration of the synthesis of liver glycogen following its oral administration.<sup>61, 60, 61</sup> Moreover, additional evidence is available from the experiments of Deuel and associates<sup>52</sup> where the increase in liver glycogen following the administration of tripropionin exceeds to a considerable extent that obtained after the feeding of triacetin or tributyrin. In the latter cases only the glycerol is utilizable for glycogenesis. With tripropionin, on the other hand, the higher values can best be explained as due to the conversion to liver glycogen of the fatty acid set free as well as of the glycerol.

The longer-chain fatty acids having an odd number of carbon atoms have also been shown to be capable of being transformed to carbohydrate. Three of the five carbon atoms of valeric acid are changed to D-glucose in the phlorizinized dog.<sup>62</sup> Their convertibility to sugar has likewise been proved by the demonstration of glycogen formation after feeding them as the sodium salts to rats (valeric, heptanoic and pelargonic acids<sup>61</sup>), or as their ethyl esters (valeric, heptanoic, pelargonic and undecylic acids<sup>61</sup>). In addition, the feeding of trivalerin and triheptanoin is followed by values for liver glycogen which far exceed that traceable to the glycerol moiety or given by the comparable even-chain triglycerides tributyrin, tricaproin or tricaprylin, where only the glycerol portion of the molecule is available for glycogenesis.<sup>52</sup>

Although the evidence is unequivocal that the odd-numbered fatty acids are converted to glycogen when fed as such or as the triglyceride,

(59) A. I. Ringer, *J. Biol. Chem.*, **12**, 511 (1912-13).

(60) H. C. Eckstein, *J. Biol. Chem.*, **102**, 591 (1933).

(61) J. S. Butts, H. Blunden, W. Goodwin and H. J. Deuel, Jr., *J. Biol. Chem.*, **117**, 131 (1937).

(62) A. I. Ringer, *J. Biol. Chem.*, **14**, 43 (1913-14).

it should be pointed out that these products are almost exclusively synthetic and that they form no part of animal fat or of the food which we ordinarily consume. Their conversion to glycogen, therefore, cannot be construed as indicating that natural fats are sources of D-glucose.

### 3. *Non-availability of Even-chain Fatty Acids as Sources of Carbohydrate*

Although there are some reports to the contrary, the evidence on the whole would seem to indicate that the straight chain fatty acids with an even number of carbon atoms are entirely ineffective as sources of carbohydrate. With the possible exception of acetic acid, the administration of the even-numbered fatty acids uniformly gives rise to a ketonuria,<sup>63, 64</sup> while it has been demonstrated repeatedly that substances convertible to carbohydrate counteract an existing ketonuria.

Although glycogenesis has been shown to follow the administration of some synthetic triglycerides of even-numbered fatty acids, it was shown above that this only occurs with triglycerides incapable of storage in the animal body, and then only to an amount corresponding to that which would be produced by the glycerol moiety of the fat.

*a. Experiments on Acetic Acid.* According to the classical  $\beta$ -oxidation theory based on the experiments of Knoop<sup>65</sup> and Dakin<sup>66</sup> with phenyl-substituted acids, acetic acid should be formed when fat is completely metabolized. If one could demonstrate that this acid is an intermediate compound formed in the degradation of the fat molecule, and if it could also be shown that it is convertible to carbohydrate, one would have a logical basis for believing that natural fats are convertible to carbohydrate. However, to neither of these questions can a positive answer be given.

In the first place, Stadie, Zapp and Lukens<sup>67</sup> failed to obtain any evidence for the production of acetic acid or any other steam-volatile acids in liver slices of depancreatized cats. These authors state further that no evidence could be found in the literature for the production of acetic acid in the livers of normal or diabetic animals except for the report of Cook and Harrison.<sup>68</sup> They believe that the acetic acid, which

(63) J. S. Butts, C. H. Cutler, L. Hallman and H. J. Deuel, Jr., *J. Biol. Chem.*, **109**, 597 (1935).

(64) H. J. Deuel, Jr., L. F. Hallman, J. S. Butts and S. Murray, *J. Biol. Chem.*, **116**, 621 (1936).

(65) F. Knoop, *Beitr. chem. Physiol. Pathol.*, **6**, 150 (1904).

(66) H. D. Dakin, *J. Biol. Chem.*, **6**, 221 (1909).

(67) W. C. Stadie, J. A. Zapp, Jr. and F. D. W. Lukens, *J. Biol. Chem.*, **137**, 75 (1941).

(68) R. P. Cook and K. Harrison, *Biochem. J.*, **30**, 1640 (1936).



Cook and Harrison reported, can be explained as originating from the oxidizing agents used in the course of the analyses.

On the other hand, Geelmuyden<sup>69</sup> has reported that acetic acid is converted to D-glucose in phlorizinized dogs when administered along with a carbohydrate diet, but the evidence can hardly be regarded as conclusive. For example, the D:N ratios<sup>69a</sup> were as follows in one experiment: 4th day, 7.6; 5th day, 6.8; 6th day (acetic acid administered), 6.6; and the 7th day, 7.0. The glucose excretion totaled 57.0, 55.1, 58.0 and 65.3 g., respectively, on these days. In contrast with these, Ringer and Lusk,<sup>70</sup> and also Milhorat and Deuel,<sup>71</sup> found no evidence of any change of administered acetate to D-glucose in the *fasted* phlorizinized dog. Geelmuyden<sup>69</sup> has suggested that acetic acid is not immediately metabolizable and that its conversion to D-glucose may be delayed as long as twenty-four hours. This is a difficult hypothesis to accept for a substance that is as readily metabolized as the acetic acid molecule and it is without precedent in a wide range of carbohydrate intermediates and amino acids which have been studied by the glycogen or phlorizin technic in the past years. Ponsford and Smedley-Maclean,<sup>72</sup> as well as Majer and Reisner,<sup>73</sup> found no glycogen formation in rats that were fed fats containing acetic acid. Similar results are reported by Stoehr<sup>74</sup> for fasted rats although slightly higher values are reported when the acetate was accompanied by a high D-glucose feeding. Likewise Clutterbuck<sup>75</sup> has failed to demonstrate any D-glucose originating from acetic acid in minced tissues of rabbits. The only conflicting evidence is that of Thunberg<sup>76</sup> who found that succinic acid originated from acetic acid in the animal organism. Succinic acid has been proved by many workers to be a D-glucose-former.<sup>77</sup>

It is evident that even if acetic acid could be demonstrated as an intermediate in fat oxidation, the experimental data are quite preponderant in indicating that it would not be a D-glucose-former.

*b. Experiments on Butyric Acid.* Butyric acid is regarded by most investigators as an intermediate in fat oxidation; any demonstration of its effectiveness as a source of glycogen should afford convincing proof

(69) H. C. Geelmuyden, *Skand. Arch. Physiol.*, **40**, 211 (1920).

(69a) The D:N ratio is the ratio between urinary D-glucose (dextrose) and urinary nitrogen.

(70) A. I. Ringer and G. Lusk, *Z. physiol. Chem.*, **66**, 106 (1910).

(71) A. T. Milhorat and H. J. Deuel, Jr., *J. Biol. Chem.*, **78**, 299 (1928).

(72) A. P. Ponsford and I. Smedley-Maclean, *Biochem. J.*, **26**, 1340 (1932).

(73) E. H. Majer and H. Reisner, *Biochem. Z.*, **263**, 340 (1933).

(74) R. Stoehr, *Z. physiol. Chem.*, **217**, 141 (1933).

(75) P. W. Clutterbuck, *Biochem. J.*, **21**, 512 (1927).

(76) T. Thunberg, *Skand. Arch. Physiol.*, **40**, 1 (1920).

(77) A. I. Ringer, E. M. Frankel and L. Jonas, *J. Biol. Chem.*, **14**, 539 (1913-14).

that the change of fat to carbohydrate is a possible transformation in the animal organism. However, butyric acid itself has not been conclusively demonstrated to be an intermediate product in fat metabolism although undoubtedly it may be such. On the other hand, there is no question that the closely related "ketone bodies" originate directly from the longer-chain acids having an even number of carbon atoms.<sup>62, 63</sup>

The evidence seems to be quite one-sided in indicating that no glycogen originates after the oral administration of butyrate to *fasted* rats. When the butyric acid was fed as its sodium salt<sup>51, 60, 73</sup> or as the ethyl ester,<sup>61</sup> no glycogen was formed in the liver. However, in experiments where the butyrate was fed with carbohydrate, there are some reports that liver glycogen was produced in larger amount than would have resulted had the sugar alone been fed. Such results are reported by Stoehr,<sup>74</sup> by Dziewiatkowski and Lewis,<sup>78</sup> as well as by Buchanan, Hastings and Nesbett.<sup>79</sup> The latter investigators employed radioactive carbon in the carboxyl group as a tracer. The liver glycogen contained more of the radioactive element than would have been expected to have resulted from the bicarbonate formed from the carboxyl of the butyric acid. However, the amount of butyrate calculated as being transformed to glycogen amounted to less than 5% of the butyrate absorbed. Dziewiatkowski and Lewis do not necessarily interpret their results as indicating proof of the change of butyrate to sugar. These investigators suggest that possibly "the butyrate is oxidized readily and thus protects the liver glycogen so that glycogenolysis is diminished or that under the conditions of these experiments butyric acid contributes to the synthesis of liver glycogen." In a further study of the effect of butyrate feeding on unfasted rats and rats simultaneously fed D-glucose, Deuel and co-workers<sup>80</sup> have been unable to detect any augmentation of glycogen in the livers of rats fed butyrate in addition to D-glucose over those which received D-glucose alone. Similar results were obtained with unfasted rats fed butyrate as compared with rats which received no supplementary feeding. Although the possibility of some conversion of butyric acid to liver glycogen could not be completely eliminated in the above work, it is clear that this occurs at most to only a limited extent. It seems much more logical to assume that instances where increased liver glycogen have been noted should be ascribed to a sparing action of the butyrate on glycogenolysis rather than to a glyconeogenesis from the butyrate molecule.

(78) D. D. Dziewiatkowski and H. B. Lewis, *J. Biol. Chem.*, **153**, 49 (1944).

(79) J. M. Buchanan, A. B. Hastings and F. B. Nesbett, *J. Biol. Chem.*, **150**, 413 (1943).

(80) H. J. Deuel, Jr., C. Johnston, Margaret G. Morehouse, H. S. Rollman and R. J. Winzler, *J. Biol. Chem.*, **157**, 135 (1945).

Other evidence which has been postulated as proof for the transformation of butyrate to D-glucose has been based on liver perfusion experiments. Blixenkrone-Møller<sup>81</sup> presented evidence for the conversion of butyrate to glycogen when butyrate was artificially perfused through a cat liver. It was suggested in his experiments that only 20% was transformed to ketone bodies while the rest was converted to D-glucose. This is contrary to the implications of the experiments of Bobbitt and Deuel<sup>82</sup> where it was shown that although ketone bodies were formed when liver slices from fasting rats were immersed in a butyrate medium, the amount of ketones recoverable was less and the quantity of butyrate which disappeared was more when glycogen was also present in the medium. Haarmann and Schroeder<sup>83</sup> have also reported that several tissues, including the liver, have the power to transform butyric acid to D-glucose. They suggest the following pathway: butyric acid, crotonic acid,  $\beta$ -hydroxy-butyric acid, acetoacetic acid, dihydroxybutyric acid, dihydroxycrotonic acid, diketobutyric acid, methyl glyoxal, pyruvic acid, and sugar. Weil-Malherbe<sup>84</sup> reported that kidney slices (but not liver slices) are able to form additional reducing material in an acetoacetate medium. He proved that this was D-glucose by the characteristic skatole test, fermentation and the osazone reaction<sup>85</sup> but he is at variance with Haarmann and Schroeder in stating that D-glucose does not originate after dihydroxybutyric acid but that acetol is the substance formed. Pyruvic acid is believed to be the intermediate compound. Stadie, Zapp and Lukens,<sup>86</sup> on the other hand, have failed to confirm either investigator. They point out that the newly formed fermentable substances in the experiments of Weil-Malherbe "from acetoacetate over control slices without acetoacetate was (by our calculation)  $4.0 \pm 2.1$  micromoles of wet tissue per g. per hour. This amount is small [compare 50 micromoles in the case of pyruvate (Benoy and Elliott (1937))], but the mean value is hardly significantly different from 0. Moreover, no lactic acid determinations were reported; hence, lactic acid as a possible precursor of the new carbohydrate cannot be excluded." It was shown by Stadie and associates<sup>86</sup> that considerable fermentable carbohydrate can be syn-

(81) N. Blixenkrone-Møller, *Z. physiol. Chem.*, **252**, 117, 137 (1938).

(82) B. G. Bobbitt and H. J. Deuel, Jr., *J. Biol. Chem.*, **143**, 1 (1942).

(83) W. Haarmann and E. Schroeder, *Biochem. Z.*, **296**, 35 (1938).

(84) H. Weil-Malherbe, *Biochem. J.*, **32**, 1033 (1938).

(85) H. Weil-Malherbe, *Biochem. J.*, **32**, 2276 (1938).

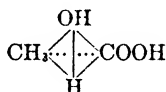
(86) W. C. Stadie, J. A. Zapp, Jr. and F. D. W. Lukens, *J. Biol. Chem.*, **137**, 63 (1941).

thesized from salts of L(*dextro*)-lactic acid.<sup>86a</sup> by liver slices from diabetic cats.

Finally, the antagonism between the urinary ketones and ingested carbohydrate would seem to speak against a conversion of butyrate to sugar. The administration of butyric acid either as the sodium salt or as the ester to fasting rats has repeatedly been found to result in the excretion of ketone bodies in the urine,<sup>87-98</sup> a phenomenon which can be partially prevented by the administration of as small an amount of D-glucose as 25 mg. per 100 sq. cm. of rat surface area twice daily. A ketonuria also results after the feeding of sodium acetoacetate<sup>99, 100</sup> as well as after  $\beta$ -hydroxybutyrate or crotonic acid.<sup>101</sup> The oral administration of sodium butyrate to fasting rats has been frequently used by the laboratory of the present authors and others for the experimental production of a ketonuria. The fact that this phenomenon is counteracted by minimum amounts of D-glucose would seem to offer satisfactory proof that butyric acid is not a D-glucose-former.

*c. Experiments on Longer Even-chain Fatty Acids.* The longer even-chain fatty acids are likewise not to be classed as glycogenic agents.

(86a) The lactic acid that is a normal constituent of animal tissue is the dextro-rotatory form and its alkali salts are levorotatory; its configuration is



and it is here designated L(*dextro*)-lactic acid. Its salts are L(*levo*)-lactates. L is the configurational symbol in the Fischer-Rosanoff system for the sugars and the words *dextro* and *levo* denote the direction of rotation of the substance that is particularly specified.

(87) H. J. Deuel, Jr., L. F. Hallman and S. Murray, *J. Biol. Chem.*, **124**, 385 (1938).

(88) H. J. Deuel, Jr., L. F. Hallman, S. Murray and J. Hilliard, *J. Biol. Chem.*, **125**, 79 (1938).

(89) C. Johnston and H. J. Deuel, Jr., *J. Biol. Chem.*, **149**, 117 (1943).

(90) C. E. Vaniman and H. J. Deuel, Jr., *J. Biol. Chem.*, **152**, 565 (1944).

(91) J. S. Butts, M. S. Dunn and L. F. Hallman, *J. Biol. Chem.*, **112**, 263 (1935-36).

(92) J. S. Butts, H. Blunden and M. S. Dunn, *J. Biol. Chem.*, **119**, 247 (1937).

(93) J. S. Butts, H. Blunden and M. S. Dunn, *J. Biol. Chem.*, **120**, 289 (1937).

(94) J. S. Butts, M. S. Dunn and L. F. Hallman, *J. Biol. Chem.*, **123**, 711 (1938).

(95) J. S. Butts, H. Blunden and M. S. Dunn, *J. Biol. Chem.*, **124**, 709 (1938).

(96) J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, **139**, 963 (1941).

(97) J. S. Butts and R. O. Sinnhuber, *J. Biol. Chem.*, **140**, 597 (1941).

(98) L. F. Remmert and J. S. Butts, *J. Biol. Chem.*, **144**, 41 (1942).

(99) J. S. Butts, *J. Biol. Chem.*, **105**, 87 (1934).

(100) J. S. Butts and H. J. Deuel, Jr., *J. Biol. Chem.*, **100**, 415 (1933).

(101) H. D. Blunden, Abstracts of Dissertations, University of Southern California Press, Los Angeles, p. 52 (1938).

Completely negative results on the formation of liver glycogen were obtained when the sodium salts of caproic or caprylic acid<sup>61, 60</sup> were fed, as well as after the administration of the ethyl esters of caproic, caprylic, capric, myristic or oleic acids or methyl laurate.<sup>61</sup> Caproic acid was found to be ineffective as a D-glucose-former in the phlorizinized dog.<sup>69</sup>

#### 4. Experiments on Fat-feeding

Several investigators, as Takao,<sup>102</sup> Burn and Ling,<sup>103</sup> and Magnusson,<sup>104</sup> have reported high values for liver glycogen following fat feeding. Burn and Ling obtained a maximum figure as high as 5% for liver glycogen in rats after the administration of fat over a ninety-six hour period. On the other hand, these results are balanced by a number of studies where negative results have been obtained.<sup>67, 105-107</sup> Gregg<sup>57</sup> found some increase in liver glycogen after the ingestion of butter but ascribes its origin to the glycerol of the fat. More recently, Gemmill and Holmes<sup>68</sup> have reported that the prolonged feeding of butter to fasted rats was first followed by a drop in liver glycogen to zero, but after the first day of butter feeding the levels of liver glycogen were considerably increased and had reached about 1% in the rats killed after a week. However, it is possible to explain the high results obtained where butter is used without resorting to the hypothesis of glycconeogenesis from fat. Since butter fat contains an appreciable amount of tributyrin, the glycerol moiety presumably is not recombined with the butyrate portion after absorption and is therefore available for conversion to carbohydrate for deposition as liver glycogen.<sup>52</sup> It should be of considerable interest to extend the above experiments by feeding fats which do not contain the glycerides of the short-chain acids. Also, there is no proof that the liver glycogen could not have arisen from protein. In fasting (where conditions are quite similar to fat feeding), there is first a lowering of nitrogen excretion but after several days this excretion frequently exceeds the pre-fasting level<sup>108</sup> until the more labile deposits become exhausted. Likewise, it is possible that variations in sex might explain the irregular results of Gemmill and Holmes,<sup>68</sup> since on fasting it is known that male rats possess much higher glycogen stores than female rats.<sup>109</sup>

(102) T. Takao, *Biochem. Z.*, **172**, 272 (1926).

(103) J. H. Burn and H. W. Ling, *Quart. J. Pharm. Pharmacol.*, **2**, 1 (1929).

(104) R. Magnusson, *Skand. Arch. Physiol.*, **55**, 279 (1928).

(105) M. G. Bodey, H. B. Lewis and J. F. Huber, *J. Biol. Chem.*, **75**, 715 (1927).

(106) E. Greisheimer, *J. Nutrition*, **4**, 411 (1931).

(107) J. C. Krantz, Jr. and C. J. Carr, *J. Pharmacol.*, **41**, 83 (1931).

(108) H. J. Deuel, Jr. and M. Gulick, *J. Biol. Chem.*, **96**, 25 (1932).

(109) H. J. Deuel, Jr., M. Gulick, C. F. Grunewald and C. H. Cutler, *J. Biol. Chem.*, **104**, 519 (1934).

If butter rather than butterfat were employed, the carbohydrate in the liver may have arisen from the protein and lactose contained in it. Although after fat feeding, values of liver glycogen not exceeding those of the fasting controls have consistently been obtained by Deuel and co-workers<sup>61</sup> in experiments with long-chain triglycerides, the maximum duration of these experiments was only forty-two hours.

### 5. *Miscellaneous Experimental Evidence that Fat is Converted to Carbohydrate*

a. *D:N Ratio.* The ratio between urinary D-glucose (dextrose) and nitrogen (D:N ratio) in phlorizinized dogs usually reaches a constant value by the fourth day of phlorizinizing and fasting, after the extra D-glucose from the stored carbohydrates has been excreted. Stiles and Lusk<sup>110</sup> believed that the value of 3.65:1 accurately represented the ratio under ideal conditions, although most workers have usually regarded values between 3.9 and 3.3 as acceptable ones in experiments where this technic was to be employed. When fat metabolism was largely increased in the fasting, completely phlorizinized dog, either by shivering or by exercise on a treadmill, Lusk<sup>111</sup> found no change in the D:N ratio provided all residual glycogen had previously been flushed out of the liver. Were fat a source of D-glucose, much larger quantities of sugar should have been produced and excreted in the urine when the lipid metabolism was increased by several hundred per cent. Moreover, it has also been shown that the lower D:N ratio (2.8:1) which occurs in fasting, depancreatized dogs is also unaltered when fat metabolism is largely increased by subjecting the animals to cold or to work.<sup>112-114</sup> However, after fat feeding, it was claimed by Hartogh and Schumm<sup>115</sup> that the D:N ratio is elevated and values as high as 13.0 are reported in fasting, phlorizinized dogs on the third to seventh day after the first injection of the drug. If such values could be authenticated, they would undoubtedly be a cogent proof for the transformation of fat to carbohydrate. Were all carbon in protein converted to D-glucose to the extent of 100%, 1 g. of urinary nitrogen (or 3.28 g. of protein carbon) would give rise to 8.2 g. of D-glucose. However, since the nitrogen is excreted largely as urea, a correction for the urea carbon would revise the maximum value of D-glucose to 6.5 g. per

(110) P. G. Stiles and G. Lusk, *Am. J. Physiol.*, **10**, 67 (1903-04).

(111) G. Lusk, *Am. J. Physiol.*, **22**, 163 (1908).

(112) Y. Seo, *Arch. exp'tl. Path. Pharmacol.*, **59**, 341 (1908).

(113) E. Allard, *Arch. exp'tl. Path. Pharmacol.*, **59**, 388 (1908).

(114) W. H. Chambers, H. E. Himwich and M. A. Kennard, *J. Biol. Chem.*, **108**, 217 (1935).

(115) Hartogh and O. Schumm, *Arch. exp'tl. Path. Pharmacol.*, **45**, 11 (1901).

g. of urinary nitrogen. On such a basis, any D:N ratio of a fasting dog exceeding 6.5 would prove that the D-glucose was arising from a non-protein source, which in this case would be limited exclusively to fat. However, Lusk<sup>116</sup> strenuously denies the accuracy of the data of Hartogh and Schumm,<sup>115</sup> and implies that they are possibly to be traced to inaccuracy in the determinations of urinary nitrogen. Hartogh and Schumm report values of 4.89 and 5.17 g. for urinary nitrogen on a 55 kg. dog on the third and seventh day, respectively, of phlorizin injection. Lusk<sup>116</sup> states: "Contrast these nitrogen values with those obtained by Mandel and Lusk<sup>117</sup> from a dog weighing 40 kg. which on the seventh day of fasting and the fourth day of phlorizin, eliminated 29.3 g. nitrogen and 107.7 g. sugar, a D:N of 3.61." Lusk denies that the figures of Hartogh and Schumm can be reproduced under proper experimental conditions.<sup>118</sup> He further states that he has never observed an increase in D:N ratio after the administration of fat to a phlorizinized dog. Takao<sup>102</sup> supports this conclusion and it is also in line with the experience of the present author.<sup>119</sup>

Soskin<sup>120</sup> has reported experiments on three depancreatized dogs where the administration of lecithin in two cases and olive oil in the third experiment increased the D:N ratio. On this basis it is suggested that the "extra sugar" is obtained from fatty acids. However, there were 10 tests where no extra sugar was eliminated after the administrations of 50 g. of fat. Also, the D:N ratio of the positive experiment reported on the day previous to the administration of lecithin was 6.21, a figure which would scarcely indicate that a complete flushing out of carbohydrate stores had already occurred. In six of the negative tests, the D:N values varied from 2.99 to 2.68, which are in excellent agreement with the postulated value of 2.8:1 as suggested by Minkowski.<sup>121</sup> Likewise it should be noted that Page and Young<sup>122</sup> have been unable to confirm the observations of Soskin.

Grafe and Wolf<sup>123</sup> have also reported exceedingly high D:N ratios (exceeding 10:1) in diabetic patients on an exclusive protein-fat diet. Lusk<sup>124</sup> suggests that such ratios may be the result of surreptitious in-

(116) G. Lusk, ref. 10, p. 639.

(117) A. R. Mandel and G. Lusk, *Am. J. Physiol.*, **16**, 129 (1906).

(118) G. Lusk, *Ergeb. Physiol.*, **12**, 315 (1912).

(119) H. J. Deuel, Jr., H. E. C. Wilson and A. T. Milhorat, *J. Biol. Chem.*, **74**, 265 (1927).

(120) S. Soskin, *Biochem. J.*, **23**, 1385 (1929).

(121) O. Minkowski, *Arch. expil. Path. Pharmacol.*, **31**, 85 (1893).

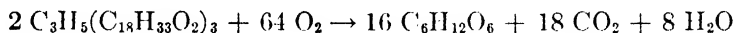
(122) I. H. Page and F. G. Young, *Biochem. J.*, **26**, 1528 (1932).

(123) E. Grafe and C. G. L. Wolf, *Deut. Arch. klin. Med.*, **107**, 201 (1912).

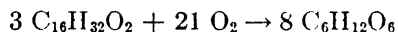
(124) G. Lusk, ref. 10, p. 640.

gestion of carbohydrate food which is greatly desired by the diabetic. He further points out that similar high ratios were obtained on diabetic patients by Greenwald as well as by DuBois. However, when the patients were confined to a private room where the food could be checked, ratios approaching 3.65:1 were immediately obtained.

*b. Respiratory Quotient.* The situation as regards the respiratory quotient (R. Q.) is reversed from what occurs when carbohydrate is transformed to fat. Were an oxygen-poor substance like fat changed to an oxygen-rich product like carbohydrate, a considerable quantity of oxygen would be required; inasmuch as a complete oxidation of the newly synthesized carbohydrate probably would not occur, a corresponding elimination of additional carbon dioxide would not be obtained. Therefore, when carbohydrate is being formed from fat, one should expect that the R. Q. would be depressed below the values normally found. Pembrey<sup>125</sup> has calculated a theoretical R. Q. of 0.28 for the change of triolein into D-glucose, based on the following equation.



Lusk<sup>126</sup> has suggested the following empirical equation for the conversion of palmitic acid to D-glucose.



The greater the proportion of the total metabolism which is concerned with the change fat  $\rightarrow$  carbohydrate, the more the R. Q. may be expected to be depressed below the fasting non-protein R. Q. of 0.707 which is usually regarded as the minimal normal value.

Many of the low R. Q.'s which have been reported in the literature may be explainable because of inaccuracies in the determination of oxygen. This is particularly true when a closed circuit apparatus is employed, where any leak in the system will result in an abnormally high value for oxygen with a resulting low R. Q.

Pembrey<sup>125</sup> has found values for the R. Q. of hibernating marmots as low as 0.55 while Voit<sup>127</sup> reported a minimum value of 0.33. In all cases where these values were obtained, the actual quantities of oxygen and carbon dioxide determined were extremely small and it is possible that inaccuracies could be traceable to analytical difficulties. It should be pointed out that most of Pembrey's values for R. Q.'s in the hibernating marmots are approximately 0.70 or above. Irregularities in breathing

(125) M. S. Pembrey, *J. Physiol.*, **27**, 66 (1901-02).

(126) G. Lusk, ref. 10, p. 671.

(127) C. Voit, *Z. Biol.*, **14**, 57 (1878).



(most animals exhibited a Cheyne-Stokes respiration) with consequent upset in the acid-base balance could easily result in a portion of the carbon dioxide being retained, with the consequence that the respiratory exchange over a short period would not be an accurate index of the carbon dioxide production and oxygen utilization. It has also been suggested that irregularities might result because of differential changes in the proportion of carbon dioxide and oxygen dissolved in the colder blood.<sup>128</sup> Although the solubility of both gases is increased at lower temperatures, the increase in solubility of carbon dioxide is greater. A greater retention of this gas would result in a lowering of the R. Q.

DuBois<sup>129</sup> has listed approximately 100 low R. Q.'s in the literature, which were obtained in laboratories where the technic was known to be thoroughly good. These were chiefly measured on diabetics although several were obtained on normal men receiving high-fat diets and on obese patients on low-calorie regimens. In the evaluation of these data, Richardson<sup>5</sup> notes that those having a R. Q. below 0.65 are in a minority, the lowest value recorded being 0.64. Since the experimental error, as found from checks on the respiration calorimeter when alcohol is burned therein, may amount to as much as  $\pm 0.02$ ,<sup>129</sup> it is not considered that even the lowest authenticated value may represent a conversion of fat to carbohydrate. In Joslin's series of 113 diabetics<sup>130</sup> only 9 subjects (18 observations) had a R. Q. below 0.70. Lusk<sup>131</sup> has reported a R. Q. of 0.687 for a fasting phlorizinized dog. Hawley<sup>132</sup> also obtained a similar result. In human subjects undergoing exercise, Stewart, Gaddie, and Dunlop<sup>133</sup> could find no evidence of a conversion of fat to carbohydrate on the basis of changes of R. Q.

If allowance is made for the fact that much of the carbohydrate formed from protein, which is oxidized in the non-diabetic, may escape metabolism by the diabetic and be excreted in the urine, the protein R. Q. for the diabetic should be 0.632 instead of the normal value of 0.802.<sup>134</sup> The R. Q. for fat is also altered because of the failure of a complete combustion of this foodstuff, due to the attendant ketonuria.

(128) E. Grafe, "Die pathologische Physiologie des Gesamtstoff-und Kraftwechsels bei der Ernährung des Menschen," J. F. Bergmann, Munich (1923).

(129) E. F. DuBois, "Basal Metabolism in Health and Disease," Lea and Febiger, Philadelphia, 3rd ed., p. 280 (1936).

(130) E. P. Joslin, "Diabetic Metabolism with High and Low Diets," Carnegie Institution, Washington, Publication No. 323 (1923).

(131) G. Lusk, ref. 10, p. 670.

(132) E. E. Hawley, *Am. J. Physiol.*, 101, 185 (1932).

(133) C. R. Stewart, R. Gaddie and D. M. Dunlop, *Biochem. J.*, 25, 733 (1931).

(134) G. Lusk, *Arch. Internal Med.*, 15, 939 (1915).

Allen and DuBois<sup>135</sup> have calculated that the R. Q. of 0.707, obtainable when fat is completely oxidized, would be lowered to 0.669 if the  $\beta$ -hydroxybutyric acid were not metabolized. If more than one molecule of the ketone bodies is produced from one molecule of the fatty acid, as has been suggested,<sup>63</sup> the R. Q. would be further lowered. This may be further complicated by a resulting upset in acid-base balance.

Another possible explanation for the low R. Q.'s obtained after high-fat diets over short periods by Hawley, Johnson and Murlin<sup>136</sup> is that in the oxidation of fatty acid chains the uptake of oxygen may outrun the production of carbon dioxide and thus lower the R. Q. Such a process of desaturation would remove the hydrogen but would not produce any carbon dioxide.

There are no convincing experimental data, based on respiratory quotients, which support the hypothesis that fatty acids are convertible to sugar. Contrasted with the marked alterations which have been observed when carbohydrate is being changed to fat, the decreases in R. Q. below the level of 0.707 for fat, which should be observed if fat were changing to carbohydrate, are very small and can be partially if not entirely explained by alterations in the R. Q. of the metabolized protein and fat, due to their incomplete oxidation.

*c. The Relation of Epinephrin to the Change of Fat to Carbohydrate.* Eppinger, Falta and Rudinger<sup>137</sup> suggested that diabetes is caused by an overproduction of sugar from fat, which process they assumed to result from an increased stimulation of adrenal secretion. They suggest also that epinephrin inhibits insulin production. These investigators cite as evidence for this theory the largely increased sugar output by depancreatized dogs when epinephrin is administered. However, such increased sugar excretion will not occur if the glycogen reserves in the liver are first exhausted, as proved by Ringer<sup>138</sup> on a phlorizinized dog and confirmed by others.<sup>139-141</sup> Kramer, Marker and Murlin<sup>142</sup> report similar results on depancreatized dogs while no glycosuria occurs in normal animals on the injection of epinephrin after the glycogen supply is exhausted.<sup>143</sup>

(135) F. M. Allen and E. F. DuBois, *Arch. Internal Med.*, **17**, 1010 (1916).

(136) E. E. Hawley, C. W. Johnson and J. R. Murlin, *J. Nutrition*, **6**, 523 (1933).

(137) H. Eppinger, W. Falta and C. Rudinger, *Z. klin. Med.*, **66**, 1 (1908).

(138) A. I. Ringer, *J. Exptl. Med.*, **12**, 105 (1910).

(139) R. T. Woodyatt, *J. Biol. Chem.*, **14**, 441 (1913-14).

(140) W. W. Palmer, *J. Biol. Chem.*, **30**, 79 (1917).

(141) R. W. Scuffert and H. Hartmann, *Beitr. Physiol.*, **2**, 199 (1924).

(142) B. Kramer, J. Marker and J. R. Murlin, *J. Biol. Chem.*, **27**, 499 (1916).

(143) F. Hildebrandt, *Arch. exptl. Path. Pharmacol.*, **88**, 80 (1920).

The role of epinephrin in the formation of carbohydrate from fat has more recently been revived by Chaikoff and Weber<sup>144</sup> who concluded from experiments on fasting, depancreatized dogs that more D-glucose originated as a result of repeated injections of epinephrin than could be accounted for from the carbohydrate reserves, and from the glycerol and protein metabolized. These authors therefore postulated that the extra D-glucose could only have arisen from fatty acids. In fact, Macleod<sup>32</sup> suggests that these experiments prove "irrefutably the derivation of sugar from fatty acid, at least in diabetic animals."

However, a number of criticisms have been leveled at the interpretation of these experiments. In the first place, they do not take into consideration the muscle glycogen which Chaikoff and Weber regard as incapable of being a source of the sugar. If this source were included, the "extra sugar" in 6 of the 9 experiments could be fully accounted for on the basis of known carbohydrate precursors. These investigators, moreover, feel justified in discounting the muscle glycogen as a source of the sugar since they found no rise in blood lactic acid. However, Cori and Cori<sup>145, 146</sup> have shown that a rise in blood lactic acid occurs after administration of epinephrin to rats, concomitantly with a decrease in muscle glycogen. Also, Bollman, Mann and Wilhelmj,<sup>147</sup> in experiments where determinations of blood sugar, muscle and liver glycogen were made before and after injections of epinephrin into 4-day-fasted depancreatized dogs, found that the "extra glucose" over a D:N ratio of 2.8:1 excreted after epinephrin, was practically identical with that which would be formed from the muscle glycogen which disappeared. Similar conclusions were reached by Chambers, Himwich and Kennard,<sup>114</sup> as well as by Bachrach, Bradley and Ivy.<sup>148</sup> The last workers have calculated an average potential amount of available D-glucose in their 4-day-fasted depancreatized dogs (16 dogs) as 25.8 g. (including 13.9 g. from muscle glycogen) while actually the average extra elimination following epinephrin injection amounted to a mean of 13.2 g. They conclude that it is unnecessary to postulate gluconeogenesis from fatty acid to account for the extra D-glucose excreted after the injection of epinephrin. Rapport<sup>1</sup> also suggests that the high D:N ratios obtained by Chaikoff and Weber<sup>144</sup> would indicate that the glycogen reserves were not as nearly stabilized as the authors assume. Further, he points out that the R. Q.'s

(144) I. L. Chaikoff and J. J. Weber, *J. Biol. Chem.*, **76**, 813 (1928).

(145) C. F. Cori and G. T. Cori, *J. Biol. Chem.*, **79**, 309 (1928).

(146) C. F. Cori and G. T. Cori, *J. Biol. Chem.*, **84**, 683 (1929).

(147) J. L. Bollman, F. C. Mann and C. M. Wilhelmj, *J. Biol. Chem.*, **93**, 83 (1931).

(148) W. H. Bachrach, W. B. Bradley and A. C. Ivy, *Am. J. Physiol.*, **117**, 203 (1936).

obtained are not in harmony with the conception that fat was being converted to carbohydrate.

One is practically forced to conclude that epinephrin does not cause the formation of carbohydrate from fat. The positive experimental data would seem to be largely outweighed by the number of negative reports.

*d. The Relation of Insulin.* Since it is well known that insulin causes the deposition of liver glycogen in the diabetic animal at the same time that a decrease in blood sugar, urine sugar and blood and liver fat is occurring,<sup>149</sup> it is not surprising that these changes have been considered to be interrelated. In fact, Wertheimer<sup>150</sup> has postulated that insulin facilitates the conversion of fat to sugar. He found that glycogen appeared in the liver of phlorizinized dogs after insulin injection simultaneously with the disappearance of fat. Moreover, his dogs which had a large quantity of fat in the liver were more resistant to insulin, the blood sugar returned to normal more quickly and recovery from insulin shock was much prompter than in control animals with lower fat stores. However, these results are refuted by the report of Hawley<sup>152</sup> who concluded that "even when there was a very great need of carbohydrate to save the life of the animal, and when there were ample stores of fat or ample food fat available to meet this need, there was no gluconeogenesis from fat."

*e. Experiments with Isolated Organs.* A considerable amount of experimental data based on results obtained when isolated organs are perfused or when tissue slices or hashed tissues are allowed to remain in buffered solutions, has been brought to bear on the fat  $\rightarrow$  carbohydrate problem.

Most of the perfusion studies have been carried out on the isolated liver since it is in this organ that the change of fat to carbohydrate presumably takes place. On the basis of some of these studies, carbohydrate formation from fat has been postulated.<sup>151-155</sup> In such cases more carbohydrate has been present at the end of the perfusion experiment than could be accounted for on the basis of known carbohydrate precursors. In several cases, a decrease in the fat content of the liver was accompanied by a concomitant increase in carbohydrate. However, Gregg<sup>156</sup> has been

(149) J. J. R. Macleod, "Carbohydrate Metabolism and Insulin," Longmans, Green and Co., London, pp. 104, 163 (1926).

(150) E. Wertheimer, *Arch. ges. Physiol. (Pflügers)*, **213**, 298 (1926).

(151) H. Heller, *Acta. Med. Scand.*, **90**, 489 (1936).

(152) G. Embden and H. Salomon, *Beitr. chem. Physiol. Pathol.*, **6**, 63 (1905).

(153) L. Lattes, *Biochem. Z.*, **20**, 215 (1909).

(154) J. H. Burn and H. P. Marks, *J. Physiol.*, **61**, 497 (1926).

(155) H. Jost, *Z. physiol. Chem.*, **197**, 90 (1931).

(156) D. E. Gregg, *Am. J. Physiol.*, **103**, 79 (1932).

unable to confirm such results. In a series of experiments on the livers removed from 17 fat-fed cats and dogs and perfused by an improved technic, he found a decrease in liver glycogen as well as liver fat during the course of the experiment. He reached the conclusion that the complexity of such a system, not only from the standpoint of chemical changes involved in carbohydrate and fat metabolism, but also due to the edema of the organ, the unevenness of the distribution of sugars in the tissues and the possible origin of D-glucose from glycerol made it practically impossible to demonstrate the formation of D-glucose from fatty acid on a quantitative basis. In order to prove a synthesis of D-glucose from fat in the liver, it is necessary to take into consideration changes not only in glycogen but also in free sugar, lactic acid, urea, glycerol, and fatty acids both in the liver and in the perfusing fluid. Although these criticisms were partly answered in the experiments outlined above,<sup>152-156</sup> they have not been entirely satisfied. Richardson<sup>5</sup> has suggested that the lactic acid formed because of the anaerobic conditions to which the tissue was subjected prior to the start of perfusion, might well account for the additional D-glucose found at the end of the experiments since this would be changed back to D-glucose as soon as the perfusion with the oxygenated solution was started. Page and Young<sup>122</sup> are of the opinion that there was sufficient glycerol available in the experiments of Jost<sup>155</sup> to account for the sugar formed. Finally, Gregg<sup>156</sup> believes that the livers of the cats and dogs used were not completely glycogen-free at the start of the experiment, as was assumed in the calculations. Moreover, he has found that the composition of the lobes of the liver is not identical, so the analysis of any one lobe cannot be taken as an initial value for the whole organ.

In addition to perfusion experiments, tests with tissue slices have been cited to prove the conversion of fat to carbohydrate. Here, both changes in composition of the slices and their R. Q. have been determined and certain deductions drawn from these values. When these slices are prepared from well-fed animals, the levels of R. Q. are usually from 0.75 to 0.85, which can hardly support the hypothesis that a conversion of fat to sugar is occurring. However, when the liver slices are obtained from rats in which the carbohydrate stores are depleted, either by fasting or by a previous high-fat diet, figures for the respiratory quotient below 0.70 have been reported<sup>154, 157, 158</sup> while Dickens and Simer<sup>159</sup> found values below 0.70 only when the tests were carried out with phosphate or bi-

(157) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **171**, 381 (1926).

(158) P. Barreda, *Arch. exptl. Path. Pharmacol.*, **178**, 333 (1935).

(159) F. Dickens and F. Simer, *Biochem. J.*, **25**, 985 (1931).

carbonate-buffered Ringer's solution in place of plasma. However, it is questionable how much weight one can place on the respiratory quotient of an isolated organ. Under the highly artificial conditions obtained in these *in vitro* studies, fat may not have the R. Q. of 0.707, but a lower one due to incomplete oxidation. If only one ketone body molecule should originate per molecule of fatty acid, a value of 0.669 would be found<sup>135</sup>; if two or three molecules of ketone bodies are formed from a single fatty acid, as would seem probable from *in vivo* studies,<sup>64</sup> a still lower value for the R. Q. might readily be explained. It is, of course, true that further revisions upward may be necessary as a result of an upset in acid-base relationship. However, such alterations are seldom complete and would be considerably modified by the buffer activity of the medium.

Gemmill and Holmes<sup>58</sup> not only found a low R. Q. in the liver slices from rats which had been on high-fat diets but also have noted some increase in fermentable carbohydrate during the course of the Warburg tests. However, Cori and Shine<sup>60</sup> point out that the addition of  $\alpha$ - or  $\beta$ -glycerophosphate or glycerol to such liver slices will result in additional fermentable carbohydrate. Also Stadie, Zapp and Lukens<sup>161</sup> have accounted for the total consumption of oxygen in three main oxidative processes in the liver, namely, deamination, carbon dioxide, and ketone body formation. Stadie<sup>162</sup> states that "*there was no oxygen whatever available in the metabolism of the diabetic liver slice for this conversion.*" A similar condition was shown to obtain in liver slices of fasting, phlorizinized rats.<sup>67</sup> It was also proved by direct analysis that there was no gluconeogenesis attributable to fat by liver slices from a diabetic cat.

An alternate suggestion as to the site of the transformation of fat to carbohydrate is the fat depots. Hoffman and Wertheimer,<sup>163</sup> as well as Schur and Löw<sup>164</sup> suggest that fat is not carried in the blood but must be transformed first to glycogen and D-glucose. However, Rony, Mortimer and Ivy<sup>165</sup> give direct evidence that the sugar content of the lymph of fasting, phlorizinized dogs is less than that of normal blood or lymph. This would seem to preclude the fat depots as the site of such changes, as the lymph and blood should contain increased amounts of sugar under such conditions where the organism was relying so largely on fat as a source of energy.

(160) C. F. Cori and W. M. Shine, *Science*, **82**, 134 (1935).

(161) W. C. Stadie, J. A. Zapp, Jr., and F. D. W. Lukens, *J. Biol. Chem.*, **132**, 423 (1940).

(162) W. C. Stadie, *J. Clin. Investigation*, **19**, 843 (1940).

(163) A. Hoffman and E. Wertheimer, *Arch. ges. Physiol. (Pflügers)*, **217**, 728 (1927).

(164) H. Schur and A. Löw, *Wien. klin. Wochschr.*, **41**, 261 (1928).

(165) H. R. Rony, B. Mortimer and A. C. Ivy, *J. Biol. Chem.*, **96**, 737 (1932).

Soskin and associates<sup>166</sup> believe that whereas the normal dog obtains carbohydrate from protein and fat, only protein can be so utilized after removal of the pituitary. This accounts for the sensitivity to insulin, the low blood sugar, and the lack of ketonuria in the depancreatized-hypophysectomized dog.

#### 6. Carbohydrate Formation from Fat in Plants

Although it is evident from the above discussion that the fatty acids present in the usual vegetable or animal fats do not contribute to the carbohydrate stores in the animal body, there is ample proof that such may be the case in plants and lower organisms. This change has been confirmed in the castor bean where the R. Q. has been found to vary from 0.30 to 0.58 during the period of germination.<sup>166a</sup> This could be correlated with the disappearance of fat and the formation of carbohydrate.<sup>167</sup> There also seems to be evidence that silk worms are able to build carbohydrate at the expense of fat.<sup>168</sup>

#### 7. Discussion

It must be evident to the reader that the experimental findings, on which support for the conversion of fatty acids to D-glucose in the animal organism must necessarily be based, are subjects of violent controversy. On the other hand, no one questions that the plant possesses the power to transform fatty acids to carbohydrate in the course of its usual metabolism. One naturally has reason to inquire why a reaction of such fundamental importance should be confined exclusively to the plant kingdom.

In the animal body, there is no doubt that glycerol can be quantitatively transformed to D-glucose. Such fatty acids as propionic and other odd-chain acids likewise can serve as sources of glycogen but they do not occur in any natural food fats. Glycogenesis may be observed after feeding fats containing triglycerides composed of short chain acids incapable of deposition in the body but here the source of the carbohydrate must be from the glycerol moiety of the fat. The remaining fields are a mass of polemical papers. Reputed transformations of fatty acids to D-glucose have been based on persisting high D:N ratios of fasting or fat-fed phlorizinized, or depancreatized dogs, on low R. Q.'s of intact animals or of isolated tissues, on "extra sugar" after epinephrin injection.

(166) S. Soskin, I. A. Mirsky, L. M. Zimmerman and N. Crohn, *Am. J. Physiol.*, **114**, 110 (1935-36).

(166a) J. R. Murlin, *J. Gen. Physiol.*, **17**, 283 (1933-34).

(167) H. B. Pierce, D. E. Sheldon and J. R. Murlin, *J. Gen. Physiol.*, **17**, 311 (1933-34).

(168) E. Couvreur, *Compt. rend. soc. biol.*, **47**, 796 (1895).

tions, on analytical changes of the isolated liver indicating a concomitant lowering of the lipid and increase in the glycogen stores of that organ, and on a number of other types of experiments. Each of these postulates has been matched by contradictory experimental evidence from laboratories of high repute or by explanations of the factors responsible for the positive results. The reviewers are of the opinion that the present information must be construed as giving a negative answer to the possibility for the fatty acid  $\rightarrow$  carbohydrate change. It is hoped that new methods of attack such as those employing the carbon isotopes will give us further insight into the complex interrelationships of carbohydrate and fat.

#### IV. THE RELATION OF KETOSIS TO CARBOHYDRATE OXIDATION

The interrelationship between carbohydrate and fat metabolism becomes most evident when one considers the phenomenon of ketosis. Whenever the animal organism cannot oxidize carbohydrate or when the supply of it is too limited, ketone bodies (or acetone bodies) accumulate in the blood (ketosis) and generally are also excreted in the urine (ketonuria). The ketone bodies originate from fatty acids although it is also recognized that several of the amino acids may contribute to them. The ketone bodies consist of  $\beta$ -hydroxybutyric acid, acetoacetic acid and a secondary product, acetone. As early as 1895, Hirschfeld<sup>40</sup> recognized that the condition responsible for the ketonuria is a failure in carbohydrate oxidation. The frequently quoted statement that "fats burn in the flame of carbohydrates"<sup>169</sup> has until recently been widely accepted as indicating the relationship between the oxidation of these foodstuffs. A similar idea of a concurrent oxidation of carbohydrate and fat to prevent an incomplete oxidation of the fatty acids has also been suggested by Geelmuyden.<sup>170-171</sup>

Shaffer<sup>172</sup> first indicated that a certain quantitative ratio must obtain between the antiketogenic and ketogenic fractions if ketonuria is to be prevented. Shaffer's original conception was based on *in vitro* experiments where it was shown that acetoacetic acid could be oxidized by hydrogen peroxide in alkaline solution only when D-glucose was present.<sup>173</sup> Ultimately, it was suggested that two molecules of acetoacetic acid were destroyed for each D-glucose molecule oxidized. Factors for the potency of the various foodstuffs to supply the antiketogenic or D-glucose equiva-

(169) G. Rosenfeld, *Klin. Wochschr.*, **43**, 978 (1906).

(170) H. D. Geelmuyden, *Z. physiol. Chem.*, **41**, 128 (1904).

(171) H. D. Geelmuyden, *Z. physiol. Chem.*, **58**, 255 (1908-09).

(172) P. A. Shaffer, *J. Biol. Chem.*, **47**, 449 (1921).

(173) P. A. Shaffer, *J. Biol. Chem.*, **47**, 433 (1921).



lents were calculated, as well as the factors for the ketogenic or fatty acid equivalents. Shaffer assumed that a ketonuria would ordinarily occur when the ketogenic-antiketogenic ratio exceeded 2:1. In a masterly treatise where such computations were applied to certain reports in the literature,<sup>174</sup> unusually good agreement was found between the calculated values for the amounts of the ketone bodies which could be expected and those actually found. Woodyatt,<sup>175</sup> on the other hand, suggested that a ratio of 1 g. of D-glucose must be available for the oxidation of 1.5 g. of fatty acid, which gives a molecular ratio of 1:1. Under such conditions diabetic acidosis could be successfully prevented. Shaffer<sup>174</sup> explained the appearance of diabetic acidosis where a ratio slightly under 2:1 existed, as due to an uneven distribution of metabolites. In border line ketosis, a D-glucose molecule might be oxidized before it encountered two ketone molecules, a phenomenon not occurring where all the metabolites were confined in a test tube.

S. R. Benedict<sup>176</sup> suggested that since the theory postulated that the simultaneous oxidation of carbohydrate caused the breakdown of the ketone bodies formed in normal metabolism, the term "ketolytic" should be used in place of "antiketogenic." Thus, these two terms have come to have entirely different connotations from those originally intended.

### 1. *Theories On the Mechanism of Ketosis*

*a. Ketolysis Theory.* According to this hypothesis, the oxidation of carbohydrate or substances capable of being changed to carbohydrate causes the disappearance of ketosis and ketonuria by producing a concomitant destruction of the ketone bodies. This might be explained on the basis that a conjugation of acetoacetic acid and the carbohydrate derivative occurs whereby both are oxidized. It might also be the result of a catalytic activity exerted by the carbohydrate although the quantitative relationships between the level of ketogenic:antiketogenic ratio or fatty acid:glucose ratio and ketonuria would indicate that the action is bound up with the oxidation of finite amounts of carbohydrate.

*b. Antiketogenesis Theory.*<sup>177</sup> The proponents of this hypothesis believe that administered D-glucose is preferentially oxidized by the animal body and consequently the formation of ketone bodies is sufficiently suppressed so that the quantity produced in the liver does not exceed

(174) P. A. Shaffer, *J. Biol. Chem.*, **54**, 399 (1922).

(175) R. T. Woodyatt, *Arch. Internal Med.*, **28**, 125 (1921).

(176) G. Lusk, ref. 10, p. 665.

(177) A review of the mechanism of ketosis which supports this theory is that of E. M. MacKay, *J. Clin. Endocrinology*, **3**, 101 (1943).

the capacity of the tissues to oxidize them. This would indicate that the oxidation of acetoacetic acid and D-glucose have no direct connection.

## 2. Experimental Evidence

*a. In vitro Analogy of Coupled Reactions.* The strongest evidence for the ketolysis theory is to be found in the experiments of Shaffer.<sup>173</sup> He found that only a slight oxidation of acetoacetic acid resulted from the action of hydrogen peroxide unless D-glucose was also present. For example, after standing for sixteen hours at 20° with hydrogen peroxide, only 8% of acetoacetate had been destroyed; when D-glucose also was present, 74% disappeared in the same interval (exp. 34). In a later report<sup>178</sup> where these experiments were extended and amplified, it was found that for the ketolytic effect of D-glucose to be observed, D-glucose itself must also be oxidized. In other words, they state "*the rate of acetoacetate disappearance becomes equal to and parallels the rate of sugar oxidation; that is to say, the rate of keto acid consumption, or 'ketolysis,' is determined and limited by the rate of sugar oxidation.*" It was also found that glycolaldehyde,  $\text{HOCH}_2\cdot\text{CHO}$ , exerts a ketolytic effect and some evidence is adduced that a condensation product resulting from a Knoevenagel type of reaction between this compound and one or two acetoacetate molecules occurs by which complete oxidation is accelerated.

The experiments of West<sup>179</sup> likewise support such a conclusion. A number of condensation products where the addition took place on the  $\alpha$ -carbon of the keto acid were found to be considerably more labile than either of their parent components. Since these new molecules possessed higher reducing potentials than acetoacetic acid, such changes in the case of oxidation could be explained on an electronic basis. In a later report<sup>180</sup> the formation of a condensation product between D-glucose and acetoacetic ester was proved. From this a series of compounds were obtained, some of which were powerful reducing agents. West indicates the role that such substances may play in the oxidation of acetoacetic acid in the following statement: "*it is plausible to suppose that various oxidations may occur in the animal body in which one compound aids the oxidation of another by combination with it forming a complex more easily oxidized by the body than one or both constituents.*"

That the rate of metabolism of several components may be speeded up when they are metabolized together over what would occur if they are acted on separately is also indicated in the increased rate of alcohol

(178) P. A. Shaffer and T. E. Friedemann, *J. Biol. Chem.*, **61**, 585 (1924).

(179) E. S. West, *J. Biol. Chem.*, **66**, 63 (1925).

(180) E. S. West, *J. Biol. Chem.*, **74**, 561 (1927).

metabolism resulting from the administration of pyruvate. Westerfeld, Stotz and Berg<sup>181</sup> believe that this is due to a coupled oxidation-reduction reaction between these substances. The oxidation of  $\beta$ -keto acids has been shown to be accelerated by an enzyme, citrogenase, which causes a condensation with oxalacetic acid to form citric acid.<sup>181a</sup> Wieland and Rosenthal<sup>181b</sup> showed that this condensation also occurred in the kidney as well as in heart muscle. These conclusions have been questioned by Weil-Malherbe<sup>181c</sup> as well as by Krebs and Eggleston.<sup>181d</sup>

*b. Specific Effect of D-Glucose and of D-Glucose-precursors on Ketonuria.* The ability of substances to reduce either exogenous or endogenous ketonuria is limited to those compounds which are capable of forming liver glycogen and of producing "extra sugar" in the urine of the phlorizinized dog. The relationship is to some extent quantitative as well as qualitative. If ketolysis is the method whereby sugar prevents ketonuria, then a certain specificity in the reaction should be expected.

In addition to D-glucose, which has repeatedly been used as a standard in such studies,<sup>49, 88-98, 182, 183</sup> D-galactose,<sup>182, 183</sup> D-fructose,<sup>182</sup> lactose,<sup>99</sup> D-mannose,<sup>88</sup> and cellobiose<sup>90</sup> also show similar relationships. In addition to the sugars, the hexitols,<sup>89</sup> such D-glucose intermediates<sup>49</sup> as L(*dextro*)-lactic acid, pyruvic acid and glycerol, and D-glucose-forming amino acids,<sup>91-98, 184-189</sup> produce similar responses in the formation of liver glycogen, the production of "extra sugar" in phlorizin glycosuria and in lowering experimental ketonuria. In no case has it been shown that substances incapable of conversion to D-glucose possess the power of lowering experimental ketonuria. Thus, D(*levo*)-lactic acid,<sup>49</sup> acetaldehyde,<sup>49</sup> ethylene glycol,<sup>49</sup> and ethyl alcohol<sup>49, 87</sup> give negative results in all respects. Such data are also reported for leucine<sup>93</sup> and cystine.<sup>95</sup>

(181) W. W. Westerfeld, E. Stotz and R. L. Berg, *J. Biol. Chem.*, **149**, 237 (1943).

(181a) F. L. Breusch, *Science*, **97**, 490 (1943).

(181b) H. Wieland and C. Rosenthal, *Ann.*, **554**, 241 (1943).

(181c) H. Weil-Malherbe, *Nature*, **153**, 435 (1944).

(181d) H. A. Krebs and L. V. Eggleston, *Nature*, **154**, 210 (1944).

(182) H. J. Deuel, Jr., M. Gulick and J. S. Butts, *J. Biol. Chem.*, **98**, 333 (1932).

(183) H. J. Deuel, Jr., E. M. MacKay, P. W. Jewel, M. Gulick and C. F. Grunewald, *J. Biol. Chem.*, **101**, 301 (1933).

(184) W. K. Hall, J. R. Doty and A. G. Eaton, *Am. J. Physiol.*, **131**, 252 (1941-42).

(185) W. C. Rose, J. E. Johnson and W. J. Haines, *J. Biol. Chem.*, **145**, 679 (1942).

(186) I. Greenwald, *J. Biol. Chem.*, **25**, 81 (1916).

(187) H. D. Dakin, "Oxidations and Reductions in the Animal Body," Longmans, Green and Co., London, 2nd ed., p. 75 (1922).

(188) G. O. Sharp and C. P. Berg, *J. Biol. Chem.*, **141**, 739 (1941).

(189) A table giving the comparative D-glucose-forming activity of various amino acids when evaluated by these three methods is included in M. Sayhun, "Outline of the Amino Acids and Proteins," Reinhold Publishing Co., New York, p. 189 (1944).

The few inconsistencies that have been found are readily explicable. It was noted in a certain proportion of the tests in which ethyl alcohol or ethylene glycol was fed that a lowering of ketonuria resulted. However, this occurred only in those experiments where a concomitant rise in protein metabolism was found. In no single experiment did a lowering in ketonuria result when the protein metabolism was not elevated. Therefore, this decreased level of ketonuria was attributable to the ketolytic effect resulting from the increased catabolism of the endogenous protein. The variations in results on cystine also have a simple explanation. Whereas Dakin<sup>190</sup> obtained "extra sugar" when cysteine was fed as the sodium salt to phlorizinized dogs, Butts, Blunden and Dunn<sup>95</sup> failed to demonstrate any increase in liver glycogen or a lowering of experimental ketonuria in rats after the administration of (*levo*)-cystine in the form of a suspension. The discrepancy may be due to species difference, a variation in metabolism of cystine and cysteine, or, as seems more probable, to a conversion of cysteine to serine in the Dakin experiments as a result of action of the alkali that was used to dissolve the amino acid. In the experiments of Butts and associates, the absorption and metabolism of the suspension of cystine was proved by the large excretion of extra sulfate in the urine.

*c. The Negative Effect of Alcohol on Lowering Ketonuria.* If the antiketogenesis theory is correct, any fat-sparing substance should reduce ketonuria as effectively as an isodynamic quantity of D-glucose. However, ethyl alcohol, which can be readily metabolized and which will presumably replace an isodynamic amount of fat calories in the fasted animals, has no effect on ketonuria. Carpenter<sup>191</sup> in a critical review of the behavior of alcohol has indicated that the preponderant evidence shows not only that alcohol can be utilized as a general source of energy but also that it may serve as a source of fuel in muscle contraction.

The administration of isodynamic amounts of alcohol or D-glucose to fasting rats having an exogenous or endogenous ketonuria gave entirely opposite results. With both types of ketosis, ketonuria was practically abolished after the administration of 100 mg. of D-glucose, while the equivalent dose of alcohol had no appreciable effect.<sup>87</sup> Thus, in the group receiving sodium butyrate (exogenous ketonuria), the average ketonuria was 118.6 mg. daily per 100 g. body weight in the control group, 26.3 mg. in the D-glucose-fed rats and 108.9 mg. in those receiving alcohol. The results on the fasting endogenous ketonuria were similar, namely, 57.9 mg. daily per 100 g. of body weight (control), 8.0 mg. (D-glucose-fed) and 53 mg. (alcohol-fed). The quantity of carbohydrate that was re-

(190) H. D. Dakin, *J. Biol. Chem.*, **14**, 321 (1912-13).

(191) T. M. Carpenter, *J. Nutrition*, **6**, 205 (1933).

quired to abolish almost completely the ketonuria in the last experiments is equivalent isodynamically to only a fraction of the fat broken down. The fat that was metabolized daily was calculated as 2464 mg. in the control rats, 2407 mg. in the D-glucose-fed group and 2392 mg. in the ethanol-fed animals. Thus, *the ketone body excretion was lowered to 7% of the control level by an amount of D-glucose isodynamically equivalent to only 2.3% of the total fat catabolized.*

A similar discrepancy between the effect on ketonuria and the extent of lowering of fat metabolism can also be demonstrated in the fasting ketosis in man. Thus it was calculated<sup>87</sup> that the fat oxidized during fasting by the subject, H. J. D., on the day before and the two days following the ingestion of 75 g. of D-galactose, was 223, 209, and 219 g., respectively, although the ketonuria on the corresponding days was 8.93, 1.43 and 1.51 g.<sup>182</sup>

These data on fasting ketonuria in man as well as the earlier described tests on the exogenous and endogenous ketonuria of rats indicate that marked changes in ketonuria can occur after the administration of carbohydrate in quantities too small to decrease significantly the fat catabolism. It is difficult to harmonize such results with the antiketogenesis theory.

One suggestion to explain this discrepancy is that two pathways of fat catabolism are available and that ketone body formation is the resultant of only one type of breakdown.<sup>177</sup> This latter type, also called the "indirect fat utilization,"<sup>182</sup> occurs in the liver; the catabolism of fat in the muscle, called the "direct" method, either involves no ketogenesis or the ketone bodies are immediately utilized and no accumulation occurs.

There is adequate proof that ketone bodies originate in the liver<sup>81, 184, 192-199</sup> and that the muscles can utilize the ketone bodies in fasting or diabetic ketosis<sup>200-202</sup> as well as normally.<sup>203-205</sup> Shaw<sup>206</sup> has

- (192) G. Embden and F. Kalberlah, *Beitr. chem. Physiol. Pathol.*, **8**, 121 (1906).
- (193) H. S. Raper and E. C. Smith, *J. Physiol.*, **62**, 17 (1926-27).
- (194) I. L. Chaikoff, *J. Biol. Chem.*, **74**, 203 (1927).
- (195) N. L. Edson, *Biochem. J.*, **30**, 1862 (1936).
- (196) I. A. Mirsky, *Am. J. Physiol.*, **115**, 424 (1936).
- (197) I. A. Mirsky, *Am. J. Physiol.*, **116**, 322 (1936).
- (198) L. A. Crandall, Jr., H. B. Ivy and G. J. Ehni, *Am. J. Physiol.*, **131**, 10 (1940-41).
- (199) H. C. Harrison and C. N. H. Long, *J. Biol. Chem.*, **133**, 209 (1940).
- (200) I. L. Chaikoff and S. Soskin, *Am. J. Physiol.*, **87**, 58 (1928).
- (201) R. H. Barnes and D. R. Drury, *Proc. Soc. Exptl. Biol. Med.*, **36**, 350 (1937).
- (202) R. H. Barnes, D. R. Drury, P. O. Greeley and A. N. Wick, *Am. J. Physiol.*, **130**, 144 (1940).
- (203) W. M. Marriott, *J. Biol. Chem.*, **18**, 241 (1914).
- (204) I. Snapper and A. Grünbaum, *Biochem. Z.*, **201**, 464 (1938).
- (205) T. E. Friedemann, *J. Biol. Chem.*, **116**, 133 (1936).
- (206) J. C. Shaw, *J. Biol. Chem.*, **142**, 53 (1942).

shown that  $\beta$ -hydroxybutyrate, but not acetoacetate, can also be utilized in the mammary gland of the cow where its oxidation contributed as much as 37% to the oxygen consumption of the organ.

There is no indisputable proof that fat can be directly oxidized in the muscles. Gemmill<sup>207</sup> was unable to demonstrate any decrease in the fat present in the working muscle of the normal or phlorizinized rat. He believed that when fat is used by muscle, it is used indirectly.

The determination of the R. Q. has been used as an argument both for and against extra-hepatic oxidation of fat. Burn and Dale<sup>208</sup> have reported a R. Q. of unity for the eviscerated cat. On the other hand, the experiments based on the R. Q. of hepatectomized dogs are brought forth as positive evidence<sup>209, 210</sup> although one should be cautious on drawing too far-reaching conclusions from such unphysiological preparations as the hepatectomized animal, a limitation of which Markowitz was aware. Another source of evidence are the experiments of Himwich and Rose<sup>211</sup> where R. Q.'s of 0.7 to 0.8 have been obtained on muscle with an intact blood supply as well as on the isolated perfused muscle. When the liver is excluded from the circulation, alterations in the protein R. Q. are to be expected due to the failure of deamination, urea formation, oxidation of purines, and other similar variations in metabolism. Corrections for these changes in total R. Q. must be made before an interpretation of non-protein R. Q. is valid. These objections also apply to the experiments on eviscerated animals.

The next question which arises is whether the oxygen disappearance in the liver is sufficient to account for the fat catabolism proceeding entirely by the "indirect" method. On the basis of the oxygen utilization of liver slices, Stadie<sup>162</sup> calculated the oxygen consumption of the whole liver and concluded that this would be sufficient for the oxidation to ketone bodies of only one-third of the total fat catabolized by the whole animal under resting conditions. Under conditions of activity the organ could utilize only enough oxygen to provide for the oxidation of one-tenth of the total fat destroyed. On the other hand, these values are based on *in vitro* experiments and it is possible that the intact liver might have a sufficient oxygen utilization to account for the oxidation of fat entirely by the indirect method. The experiments of Crandall, Ivy and Ehni<sup>198</sup> would seem to bear this out since they report that hepatic ketogenesis can supply up to 57% of the total caloric requirements.

(207) C. L. Gemmill, *Bull. Johns Hopkins Hosp.*, **66**, 71 (1940).

(208) J. H. Burn and H. H. Dale, *J. Physiol.*, **59**, 164 (1924).

(209) J. Markowitz, *Am. J. Physiol.*, **83**, 698 (1927-28).

(210) D. R. Drury and P. D. McMaster, *J. Exptl. Med.*, **49**, 765 (1929).

(211) H. E. Himwich and M. I. Rose, *Am. J. Physiol.*, **88**, 663 (1929).

In any event it can be demonstrated that a large part of *administered* fatty acids pass through the ketone body stage by indirect oxidation. As much as 50% of administered butyrate and caprylate (or 100% on the basis of one ketone body fragment per molecule) have been found as ketone bodies in the urine of fasted female rats after the feeding of these acids in the form of sodium salts<sup>63</sup>; after the feeding of methyl stearate the excess ketone excretion amounted to as much as 50% of the administered ester.<sup>64</sup> After the feeding of deuterio-caproate, 83% was calculated to have been excreted as acetone bodies.<sup>212</sup> While it is true as MacKay<sup>177</sup> suggests, that this result can partly be traced to the alkali set free when the sodium salt of the fatty acid is oxidized, this explanation accounts for only a small proportion,<sup>213</sup> and the criticism is not valid for the ester.

Obviously, more direct experimental work is required on this difficult problem before we can arrive at a satisfactory conclusion on whether fat is directly metabolized in the muscle without ketone body formation.

*d. The Effect of D-Glucose on Ketonuria Resulting from Administered Acetoacetate.* Although it is difficult to present decisive proof that the lowered ketone excretion following sugar administration in cases of endogenous ketonuria may not be a resultant of the decreased production of ketone bodies in the liver, such an explanation can scarcely be valid for the effect on exogenous ketonuria produced by acetoacetate feeding. Here the supply of ketogenic material cannot be stored or withheld. If the metabolism of acetoacetic acid were not related to that of D-glucose or if it were used preferentially to D-glucose,<sup>214</sup> then its elimination should be unchanged after sugar feeding from that observed in fasting. If D-glucose were used preferentially, then the ketone body elimination should be increased when carbohydrate is fed. However, the opposite phenomenon occurs. Ketonuria can be completely suppressed after acetoacetate feeding<sup>215</sup> if carbohydrate in sufficient excess is given. Neither acetoacetate,  $\beta$ -hydroxybutyrate nor butyrate can be stored in the animal body as such; the butyrate cannot be retained as the triglyceride.<sup>65</sup> It has also been shown that deuterium from ingested deuterio-tributyrin is rapidly excreted as D<sub>2</sub>O, which would prove that the butyrate is not built up into long-chain acids but is rapidly metabolized.<sup>28</sup>

(212) Margaret G. Morehouse and H. J. Deuel, Jr., *Proc. Soc. Exptl. Biol. Med.*, **45**, 96 (1940).

(213) J. S. Butts, H. J. Deuel, Jr., and L. Hallman, *Proc. Soc. Exptl. Biol. Med.*, **32**, 898 (1934-35).

(214) E. M. MacKay and R. H. Barnes, *Proc. Soc. Exptl. Biol. Med.*, **34**, 682 (1936).

(215) J. S. Butts, "The Comparative Ketolytic Action of Glucose, Galactose and Lactose When Administered to Rats Suffering from an Artificial Ketosis," Dissertation, University of Southern California (1933).

The adherents of the antiketogenesis school have interpreted these data as indicating that D-glucose or any precursor<sup>177</sup> lowers the fasting ketosis; the administered acetoacetate can then replace the endogenous ketone bodies and be entirely oxidized by the peripheral tissues without a resulting ketonuria. Such an interpretation, however, is inconsistent with the isodynamic law. In the previously cited experiment, the daily administration of 100 mg. of D-glucose per 100 sq. cm. of surface area caused an average lowering of ketonuria of 46.4 mg. (as acetone) or 83.2 mg. as  $\beta$ -hydroxybutyrate. The quantity of fat isodynamic with 100 mg. of D-glucose is approximately 44 mg.; if only one ketone originated per fatty acid molecule, this quantity of fat so spared would account for only 15.7 mg. of ketones as  $\beta$ -hydroxybutyrate or 18.8% of that which actually disappeared. Even if multiple alternate oxidation occurs, and four ketone body fragments should originate, for each fatty acid molecule this would account for a maximum of only 75% of the butyrate which actually disappeared. It seems highly improbable that the formation of ketone bodies should proceed to any such extent to involve a 100% transformation of the fatty acids.

*e. The Effect of D-Glucose on the Rate of Disappearance of Ketone Bodies in the Intact Animal.* Another disputed question is whether the presence of D-glucose accelerates the rate of disappearance of the ketones in the intact animal. Chaikoff and Soskin<sup>200</sup> were unable to demonstrate variations in utilization of the ketone bodies after the injection of sodium acetoacetate in nephrectomized dogs which were otherwise intact or which were eviscerated, utilization values being 120 and 130 mg. of acetoacetic acid per kg. per hour. In depancreatized, nephrectomized animals the apparent utilization rate was only 8.5 mg. per kg. per hour as compared with the rate of 210 mg. in a diabetic, eviscerated dog. However, it is suggested that the low rate in the depancreatized dogs is to be attributed to the addition of ketone bodies to the blood due to the endogenous ketogenesis which was superimposed on that caused by the injected acetoacetate.

Mirsky and Broh-Kahn<sup>216</sup> found no alteration in the rate of disappearance of injected acetoacetate in fasted or carbohydrate-fed nephrectomized rabbits. In a later report,<sup>217</sup> this group also could not demonstrate any more rapid removal of injected  $\beta$ -hydroxybutyrate in fasted than in fed nephrectomized rats.

These results were criticized by Deuel and associates<sup>218</sup> who repeated

(216) I. A. Mirsky and R. H. Broh-Kahn, *Am. J. Physiol.*, **119**, 734 (1937).

(217) I. A. Mirsky, N. Nelson and I. Grayman, *J. Biol. Chem.*, **130**, 179 (1939).

(218) H. J. Deuel, Jr., L. F. Hallman, P. O. Greeley, J. S. Butts, and N. Halliday, *J. Biol. Chem.*, **133**, 173 (1940).



the experiments with the following variations. The animals were subjected to operation on the previous day in order to prevent the depressing action of ether and trauma on carbohydrate metabolism; approximately three times the quantity of  $\beta$ -hydroxybutyrate was administered since the dosages of Mirsky and associates<sup>217</sup> were within the capacity of the animal to oxidize without the aid of added carbohydrate; the material was administered intraperitoneally rather than intravenously to minimize the shock to the animal; (*levo*)- $\beta$ -hydroxybutyrate<sup>218a</sup> was used instead of the optically inactive racemic mixture; moreover, the (*levo*)- $\beta$ -hydroxybutyrate which we employed was a highly purified preparation (the unpurified commercial racemic- $\beta$ -hydroxybutyrate was found to contain an appreciable excess of base); the experimental periods were longer; and finally only rats in a satisfactory condition at the end of the test were used. A considerably higher utilization rate was found for the D-glucose-fed animals than for the fasted controls. In the seventy-five minute experiments, 40.4 mg. of the hydroxy acid was used per 100 sq. cm. of body surface per hour compared with a value of 21.7 mg. in the fasted rats. In the 150 minute tests, the average rates of utilization were 41.7 and 27.6 mg. in the fed and fasted groups respectively. The differences between these groups were found to be statistically valid.

MacKay<sup>177</sup> has accepted the negative results of Mirsky and associates<sup>217</sup> rather than the positive ones of Deuel and associates<sup>218</sup> for the following reasons. He believes on the basis of earlier work<sup>219</sup> that the alkali set free when the salt of the ketogenic acid is oxidized by the fasted rats, would itself be ketogenic while such a ketosis could not develop in the D-glucose-fed rats under similar conditions. However, such a criticism could more significantly be raised against the experiments of Mirsky and associates<sup>217</sup> in spite of the lower doses of  $\beta$ -hydroxybutyrate which they employed, since the utilization of the injected ketones in their fasted group exceeded that in the experiments of Deuel and associates (37.6 mg. as compared with 27.6 mg. calculated as  $\beta$ -hydroxybutyrate per 100 sq. cm. per hour). Moreover, the excess alkali would have been further augmented in the Mirsky tests if the commercial racemic salt was not extensively purified. Also, the quantity of alkali available in the experiments of Deuel and associates was less than if the salt alone were employed. On theoretical grounds a 2.6 M concentration of the salt would have a pH of 9.56 whereas the  $\beta$ -hydroxybutyrate solution used in the latter experiments was adjusted to a pH of 7.4.

(218a) The  $\beta$ -hydroxybutyric acid of diabetic urine is the levorotatory form and its alkali salts are likewise levorotatory.

(219) E. M. MacKay, A. N. Wick, H. O. Carne and C. P. Barnum, *J. Biol. Chem.*, **138**, 63 (1941).

In spite of the fact that the chief criticism leveled against the experiments of the present reviewers is even more applicable to the experiments of Mirsky, the quantity of alkali set free would hardly be able to provoke a ketogenesis in an amount sufficient to account for the higher quantities present in the fasted rats as compared with the D-glucose-fed animals. When sodium bicarbonate was administered to fasting male rats in dosages equivalent to twice the potential amount injected in the present experiments,<sup>213</sup> no rise in ketonuria could be observed in the succeeding twenty-four hour period while the increase in the female rats was only 5% of that obtained when an equivalent of sodium acetoacetate was given. While it is realized that results on ketonuria are not directly applicable in the present test, differences in the ketone body excretion similar to the variations found in the tissues would be expected. Moreover, the actual quantity of alkali set free per hour was only 5% of the quantity employed above in which only a minimal response was obtained. It must therefore be a negligible correction.

*f. In vitro Tests with Liver Slices.* The liver is recognized as a chief site of ketogenesis.<sup>192, 220-222</sup> Liver slices from rats are able to destroy ketone bodies under aerobic conditions<sup>223, 224</sup> although this reaction can be inhibited by malonic acid. The rate at which spontaneous ketogenesis occurs is accelerated in liver slices from fasted rats and depressed in slices from well-fed animals when the substrate contains no ketone bodies, or when butyric acid is added.<sup>82, 225</sup> Also the rate of ketolysis in liver slices (from well-fed rats, rabbits and guinea pigs) immersed in an acetoacetate substrate was found to be much greater than in preparations from fasted animals of the same species.<sup>225</sup> The greater susceptibility of the monkey to a fasting ketonuria over that of the lower animals was found to be due to a lower rate of ketolysis in the liver both under normal alimentation and during fasting. Quastel and Wheatley<sup>226</sup> found that glycogen, but not D-glucose, lowers the oxygen consumption and ketone production of fasted livers where the substrate contained butyric, crotonic or caproic acid. They have likewise noted that the addition of propionic acid to a substrate containing butyrate produced a marked depression

(220) H. E. Himwich, W. Goldfarb and A. Weller, *J. Biol. Chem.*, **93**, 337 (1931).

(221) I. Snapper, A. Grünbaum and J. Neuberger, *Biochem. Z.*, **167**, 100 (1926).

(222) M. Jowett and J. H. Quastel, *Biochem. J.*, **29**, 2181 (1935).

(223) N. L. Edson and L. F. Leloir, *Biochem. J.*, **30**, 2319 (1936).

(224) I. E. Stark and P. P. Cohen, *J. Biol. Chem.*, **123**, cxv (1938).

(225) P. P. Cohen and I. E. Stark, *J. Biol. Chem.*, **126**, 97 (1938).

(226) J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **27**, 1753 (1933).

in  $Q_{\text{acetoacetate}}$ .<sup>226a</sup> These authors attribute this result to a substrate competition between propionic and butyric acids but they also suggest that it might be ascribed to ketolysis. Quastel and Wheatley,<sup>227</sup> however, were unable to confirm these results a year later on the same or on a new sample of glycogen. In view of our present knowledge that adenosine triphosphate is required in the synthesis of glycogen from D-glucose<sup>228</sup> as well as in the oxidation of fatty acids in homogenized liver preparations,<sup>229</sup> it may well be that a deficiency of this coenzyme may have been the cause of the failure to obtain the effect with D-glucose and for the second failure with glycogen.

Quastel and Wheatley<sup>226</sup> believe their results prove antiketogenesis rather than ketolysis as the cause for the lowered ketone body formation in the liver slices with a glycogen substrate, since a lower  $Q_0$ ,<sup>229a</sup> is observed. However, Cohen and Stark<sup>225</sup> report that a greater disappearance of ketone bodies occurs when liver slices obtained from fed rats are used in an acetoacetate substrate than when the slices are obtained from fasted rats. They explain such experiments on the basis of ketolysis. That ketolysis explains such an increased rate of utilization of ketone bodies is also supported by the results of Bobbitt and Deuel.<sup>82</sup> In these experiments the rate of butyrate disappearance from the substrate was found to be increased on the addition of glycogen to the substrate and the quantity of ketone bodies recoverable was also found to be lower. This would seem to offer cogent evidence that the lowering in recoverable ketone bodies in the substrate when glycogen is present is not traceable to a decreased formation (antiketogenesis) but is the result of an augmented destruction (ketolysis). MacKay<sup>177</sup> in discussing these results has suggested the limitations in this type of experiment and states that "the large variability in their results and the very small difference in the rate of butyrate and ketone body disappearance in the liver-slice substrate with glycogen as compared with the substrate without glycogen make it difficult to evaluate the significance of their data." However, not only were the differences in quantity of butyrate which disappeared in the substrates with and without glycogen far beyond the experimental error (496  $\mu\text{g.}$  per 10 mg. liver without glycogen, 594  $\mu\text{g.}$  with glycogen or an

(226a)  $Q_{\text{acetoacetate}}$  is an index of the rate of acetoacetate formation. It is the number of cubic millimeters of carbon dioxide equivalent to the acetoacetate formed per milligram of dry tissue per hour.

(227) J. H. Quastel and A. H. M. Wheatley, *Biochem. J.*, **28**, 1014 (1934).

(228) G. T. Cori, C. F. Cori and G. Schmidt, *J. Biol. Chem.*, **129**, 629 (1939).

(229) A. L. Lehninger, *J. Biol. Chem.*, **157**, 363 (1945).

(229a)  $Q_{O_2}$  indicates the respiration rate which is obtained by dividing the amount of oxygen absorbed in a given time by the weight of the tissue taken.

increase of 20%) but the same is the case with the total acetone bodies recovered (338  $\mu$ g. per 10 mg. liver without glycogen, 278  $\mu$ g. with glycogen). The consistency of the results is indicated from the fact that in 9 of the 10 livers studied, the basal rate of butyrate disappearance does not equal the level found when glycogen and butyrate were both present in the medium. There does not appear to be any satisfactory way to explain the effect of glycogen when present in the liver<sup>82, 225, 226</sup> or when added in the substrate<sup>82, 226</sup> on the resultant ketone body concentration except on the basis of ketolysis. It would appear, therefore, that in addition to its ability in ketogenesis, the liver is also capable of causing a destruction of the ketone bodies, presumably by a process of ketolysis.

Although Edson<sup>229b</sup> postulates that carbohydrates, as well as their derivatives are antiketogenic and compete with fatty acids for oxygen in the liver (as demonstrated by the Warburg technic), his results are only inferential since they are not based on the direct determination of the fatty acid decrease. Bobbitt and Deuel,<sup>82</sup> on the other hand, found that not only was the formation of ketone bodies depressed when glycogen was added to the medium, but also that the disappearance of butyrate, as determined by direct analysis, was increased under such circumstances.

*g. The Effect of Concentration on the Rate of Oxidation of  $\beta$ -Hydroxybutyrate.* The rate of oxidation of the ketone bodies is a direct function of their concentration in the tissues. Several investigators report that the rate of such oxidation is not influenced by the quantity of D-glucose simultaneously available. Such data offer strong support for the antiketogenesis theory.

In the heart-lung preparation, the rate of utilization of  $\beta$ -hydroxybutyrate has been found by Barnes, MacKay and associates<sup>230</sup> to be proportional to its concentration and it has likewise been reported that this rate of utilization is uninfluenced by the presence of additional carbohydrate.<sup>231, 232</sup>

In the intact animal, Wick and Drury<sup>233</sup> were able to correlate the blood ketones with the rate of utilization up to a point where their oxidation accounted for approximately 90% of the oxygen consumed. Similar results have been reported on rats<sup>234</sup> and dogs.<sup>235, 236</sup> The administration

(229b) N. L. Edson, *Biochem. J.*, **30**, 1862 (1936).

(230) R. H. Barnes, E. M. MacKay, G. K. Moe and M. B. Visscher, *Am. J. Physiol.*, **123**, 272 (1938).

(231) R. H. Barnes and D. R. Drury, *Proc. Soc. Exptl. Biol. Med.*, **36**, 350 (1937).

(232) E. T. Waters, J. P. Fletcher and I. A. Mirsky, *Am. J. Physiol.*, **122**, 542 (1938).

(233) A. N. Wick and D. R. Drury, *J. Biol. Chem.*, **138**, 129 (1941).

(234) N. Nelson, I. Grayman and I. A. Mirsky, *J. Biol. Chem.*, **140**, 361 (1941).

(235) T. E. Friedemann, *J. Biol. Chem.*, **116**, 133 (1936).

(236) J. A. Dye and J. L. Chidsey, *Am. J. Physiol.*, **126**, 482 (1939).

of carbohydrate to intact animals has been reported to have little effect on the rate of removal of the ketones.<sup>216, 217, 236</sup> The experiments of the present authors with rats (see page 153) are at variance with these.

There is unanimity of opinion that increasing quantities of ketones can be metabolized by the peripheral tissues when these substances are available in higher concentrations. The results are contradictory on whether carbohydrate augments the rate of disappearance at any one concentration of  $\beta$ -hydroxybutyrate. The unknown factor in evaluating the experimental data is the comparative metabolism of the (*levo*) and (*dextro*)- $\beta$ -hydroxybutyrate. It is possible that the oxidation of carbohydrate and of the natural levorotatory isomer of  $\beta$ -hydroxybutyrate may be related while no such relationship would be evident with the unnatural dextrorotatory isomer. In the one report where the natural isomer was used,<sup>218</sup> there is a clear relation between the carbohydrate available and the amount of the hydroxybutyrate which was oxidized. In most of the other experiments where no such effects of carbohydrate were evident, racemic preparations of hydroxybutyrate were employed, although in one case,<sup>236</sup> where no relationship was found, acetoacetate was used. Another possible criticism of the experiments is that the quantities of ketones employed were unphysiological. The concentrations in most cases far exceeded the highest levels to which the cells might be subjected even under conditions of severe ketosis. The oxidation of the ketones in the presence of a plethora might follow an entirely different pathway than would occur with moderate quantities.

### 3. Discussion

As a result of the more recent work, it is apparent that the ketolysis theory as originally suggested by Shaffer should be somewhat modified. Observations such as those on the effect of concentration on the extent of oxidation of the ketones are difficult to explain on the basis of the original theory.

On the other hand, there are a number of experiments which indicate that a relationship must exist between the oxidation of the ketone bodies and sugar; these cannot be explained on the antiketogenesis theory. The strongest *in vitro* proof was originally demonstrated by Shaffer<sup>173</sup> and has since been extended by West.<sup>179, 180</sup>

Further evidence is afforded by the *in vivo* experiments where it has been shown that only D-glucose and D-glucose-precursors are able to lower ketonuria. According to the antiketogenesis theory, any readily metabolizable sources of calories should be able to suppress the oxidation of an isodynamic quantity of fat and so prevent the formation of the

ketones. Such results, however, do not occur and non-D-glucose formers such as ethyl alcohol, ethylene glycol, D(*levo*)-lactic acid, and non-glycogenic amino acids are completely ineffective.

The quantity of carbohydrate that is necessary to abolish ketonuria is far less than would be required from an isodynamic standpoint. This is particularly the case with the exogenous ketonuria produced by the administration of acetoacetate. Finally, an *in vitro* analogy to such a reaction has been found in the ketolytic effect of glycogen on liver slices.<sup>81</sup>

The most serious objection to the ketolysis theory has been the report that D-glucose is without effect on the rate of disappearance of ketones in the animal body. This has, however, been denied by one group of investigators and it is possible that the divergent results might be ascribed to the use of the natural levorotatory isomer of  $\beta$ -hydroxybutyrate rather than the racemic salt employed by others.

Another phenomenon which is difficult to interpret on the ketolysis basis is the finding that the rate of utilization of the ketones rises sharply with increased concentrations in the blood and tissues. The quantities oxidized under such circumstances apparently have no relationship to the carbohydrate utilized. In fact, they may practically exclude the oxidation of other metabolites since they have been reported to account for 90% of the total oxygen consumption at sufficiently high concentrations. However, such levels of ketones are never found normally and possibly a different relationship to carbohydrate occurs at physiological values. Likewise it is not clear whether a similar response would be expected if the natural isomer alone were administered.

It would seem that normally the oxidation of ketone bodies would proceed largely to completion in the liver by the ketolytic mechanism. Whenever the supply of carbohydrates here is sufficiently reduced, appreciable amounts of ketones then escape oxidation and pass into the blood. When the concentration of ketones becomes sufficiently elevated, a ketonuria occurs and also some ketones will be utilized by the tissues. Such a theory would largely limit the ketolysis mechanism to the liver. It would explain the specificity of the sugars in preventing ketonuria and the discrepancy between the amount of D-glucose required to prevent ketosis and the caloric value of the fat spared. It is further supported by the demonstration that the liver is capable of exhibiting ketolysis.

## V. LACTOSE AND FAT UTILIZATION

The ability of an animal to utilize fats for growth has been reported to be dependent on the nature of the dietary carbohydrate. When weanling rats were given diets high in lactose, Boutwell, Geyer, Elvehjem

and Hart<sup>237</sup> have reported that those receiving butterfat grew more than rats which received corn, coconut, cottonseed, soybean, peanut or olive oils, lard or "Crisco" (hydrogenated cottonseed oil) but this superiority was no longer found when a mixed carbohydrate ration was employed. However, the reported superiority of butterfat over vegetable fats when the lactose is present in the concentration found in skimmed milk<sup>238</sup> has not been confirmed by others<sup>239, 240</sup> for normal weanling rats nor for prematurely weaned rats.<sup>241, 242</sup> It has been suggested that the better growth observed in a number of cases with butterfat is to be explained on a basis of food preference for the butter flavor whereby the animals consumed more and consequently grew more rapidly.<sup>243</sup> There is no evidence that such diets are more efficiently utilized. There are several recent reviews on the subject of the comparative nutritive value of fats which are of interest in this connection.<sup>244-247</sup>

(237) R. K. Boutwell, R. P. Geyer, C. A. Elvehjem and E. B. Hart, *J. Nutrition*, **26**, 601 (1943).

(238) E. J. Schantz, C. A. Elvehjem and E. B. Hart, *J. Dairy Sci.*, **23**, 181 (1940).

(239) H. J. Deuel, Jr., E. Movitt, L. F. Hallman and F. Mattson, *J. Nutrition*, **27**, 107 (1944).

(240) K. M. Henry, S. K. Kon, T. P. Hilditch and M. L. Meara, *J. Dairy Research*, **14**, 45 (1945).

(241) H. J. Deuel, Jr., and E. Movitt, *J. Nutrition*, **29**, 237 (1945).

(242) L. P. Zialcita, Jr., and H. H. Mitchell, *Science*, **100**, 60 (1944).

(243) H. J. Deuel, Jr. and E. Movitt, *J. Nutrition*, **27**, 339 (1944).

(244) Anonymous, *Nutrition Revs.*, **2**, 267 (1944).

(245) H. J. Deuel, Jr., *Science*, **103**, 183 (1946).

(246) G. R. Cowgill, *Physiol. Revs.*, **25**, 664 (1945).

(247) H. J. Deuel, Jr., in A. E. Bailey, "The Chemistry and Technology of Cottonseed Products," Interscience Publishers, New York, in press.

# THE CHEMISTRY OF MUCOPOLYSACCHARIDES AND MUCOPROTEINS

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## I. INTRODUCTION

The animal body may be regarded as a highly complex machine built mainly of plastic materials with some constituent parts composed of rubber-like products, with others containing fibers of high tensile strength and with all moving parts lubricated by fluids of remarkable efficiency. The high polymers of which animal body tissues and fluids are formed are all products of cellular origin and it is therefore not surprising that closely related substances are to be found among the constituent protoplasm of plants and microorganisms.

A considerable wealth of information is available on the general chemistry of these substances but precise structural knowledge is still lacking and a wide field of research awaits the activities of the chemist. This field not only covers carbohydrate and protein chemistry, but the area lying between the two, and it also borders on the lipid field.

It is not surprising that chemists have neglected protein-carbohydrate complexes when one considers that typical examples are represented by such substances as saliva, serum, gastric mucin or better perhaps, by frog spawn mucin and the protoplasm of jellyfish! An attempt to prepare tangible amounts of homogeneous material from these sources is often disappointing, the elusive nature of the complexes being due in a large measure to their remarkable degree of hydration.

All compounds have a precise and often highly specific function to fulfil, and in animals many of them are concerned also with the protection of the body against agents of disease. The problems of immunity and of enzyme systems involve the consideration of protein-carbohydrate complexes so that structural studies in the group now need to be undertaken seriously.

The conjugated proteins involved have been classified generally as mucins, mucoids, glycoproteins, glycoids, mucoproteins, etc., and earlier work was described by Levene.<sup>1</sup> His monograph is most valuable as a record of the earlier literature and for its preparative details, but the structural formulas given in it for complex substances are misleading and often have no foundation in fact. The first attempt at classification was

made by Meyer<sup>2</sup> who includes only hexosamine-containing compounds and who considers that the mucins and mucoids of the older literature occur in nature as free polysaccharides or as protein salts. The reviewer<sup>3</sup> considered that Meyer's classification did not go far enough and suggested a system which included many other complex carbohydrates. This classification, now slightly modified, is given below. The physical and immunological properties of polysaccharides are remarkably influenced by the presence in them of even low concentrations of bound protein or peptide residues so that one appears justified in attempting to differentiate between the various states of degradation of the complexes. It must also be pointed out that in order to obtain polysaccharides in soluble form from natural sources, drastic methods, often involving the use of alkali, are generally needed. The scheme will need to be modified as knowledge of structure increases, particularly since the polysaccharides themselves, by loss of labile residues, can undergo various degrees of degradation.

It is not proposed to include the better known polysaccharides or the plant gums in the group because they can be conveniently classified separately. It must be pointed out, however, that some of these may occur naturally in firm combination with protein as instanced by the shock effect observed when solutions of crude inulin are injected into animals and by the reported isolation of desmoglecogen<sup>4</sup> and glycogen combined with myosin.<sup>5</sup>

In this classification of the compounds into mucopolysaccharides, mucoproteins, and mucolipids, it must be emphasized most strongly that since the mode of isolation of any naturally occurring high polymer influences profoundly its composition and biological properties, the difference between divisions is not always sharp and there may be some degree of overlapping.

## II. CLASSIFICATION OF PROTEIN-CARBOHYDRATE COMPLEXES

*Mucopolysaccharides* (See section IV). In general these compounds have a low but significant protein content and give reactions which are predominantly carbohydrate. Their aqueous solutions show a high viscosity and they tend to become insoluble in water after intensive dehydration.

(1) P. A. Levene, "Hexosamines and Mucoproteins," Longmans, Green and Co., London (1926).

(2) K. Meyer, *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 99 (1938).

(3) M. Stacey, *Chemistry & Industry*, **52**, 110 (1943).

(4) K. H. Meyer, *Advances in Enzymol.*, **3**, 132 (1943).

(5) E. Mystowski, *Biochem. Z.*, **276**, 240 (1935).

1. <i>Containing hexosamine and hexuronic acid</i>	<i>Identified products of acid hydrolysis of carbohydrate portion</i>
a. Sulfate-free	
(1) Hyaluronic acid (from vitreous humor, umbilical cord, synovial fluid, ovarian tumor, Group "A" hemolytic streptococcus and skin)	N-acetyl-D-glucosamine hexuronic acid (probably D-glucuronic acid)
(2) Type I pneumococcus specific polysaccharide	acetic acid hexosamine D-galacturonic acid
b. Sulfate-containing	
(1) Heparin	sulfuric acid D-glucuronic acid D-glucosamine (unacetylated)
(2) Chondroitin sulfate	N-acetyl-chondrosamine sulfuric acid D-glucuronic acid
(3) Mucoitin sulfate (from gastric mucin)	N-acetyl-D-glucosamine hexuronic acid sulfuric acid
(4) Hyaluronic acid sulfate (from the cornea)	Sulfuric acid N-acetyl-hexosamine hexuronic acid
2. <i>Containing hexosamine but no hexuronic acid</i>	
a. Chitin	N-acetyl-D-glucosamine
b. Bacterial	
(1) Pneumococcus "C" (or Group) polysaccharide	N-acetyl-amino sugar D-glucose
(2) Type IV pneumococcus specific polysaccharide	N-acetyl-amino sugar D-glucose
(3) Type XIV specific polysaccharide	N-acetyl-D-glucosamine D-galactose
c. Blood Group	
(1) Blood Group A carbohydrate (from saliva, pepsin, urine, gastric mucin, pancreas, etc.)	N-acetyl-D-glucosamine D-mannose D-galactose L-fucose
(2) Blood Groups B and O carbohydrates from human saliva and gastric mucin	hexosamine
3. <i>Containing hexuronic acid but no hexosamine</i>	
a. Type II pneumococcus specific polysaccharide	D-glucose D-glucuronic acid L-rhamnose
b. Type III pneumococcus specific polysaccharide	D-glucose D-glucuronic acid
c. Type VIII pneumococcus specific polysaccharide	D-glucose D-glucuronic acid

d. <i>Azotobacter</i> and <i>Rhizobia</i> capsular polysaccharides	D-glucose D-glucuronic acid
e. Friedlander's bacillus polysaccharides	D-glucose D-glucuronic acid
f. <i>Cytophagae</i> polysaccharide	D-glucose D-glucuronic acid
4. Containing neither hexosamine nor hexuronic acid	
a. Bacterial dextrans	D-glucose
b. Bacterial and plant levans	D-fructose
c. Yeast mannan	D-mannose
d. Luteic acid from <i>Penicillium luteum</i> Zukal	D-glucose malonic acid
e. Mold polysaccharides	D-mannose D-galactose, etc.
f. Snail mucin	D-galactose

*Mucoproteins* (See Section V).—Protein-carbohydrate compounds with relatively high protein or peptide content, the chemical reactions of which are predominantly protein, are known as mucoproteins. In general they do not coagulate on being heated in aqueous solution. All mucoproteins contain a hexosamine constituent.

The most radical change of classification in this group is the abandonment of Levene's<sup>1</sup> definition that "the carbohydrate group of all mucoproteins is conjugated with sulfuric acid."

*Identified hydrolytic products of carbohydrate residue*

1. Ovomuroid	N-acetyl-D-glucosamine, D-mannose, D-galactose
2. Serum mucoproteins	
a. Seromuroid	D-mannose, D-glucosamine
b. Seroglycoid	D-mannose, D-galactose, D-glucosamine
c. Globoglycoid	D-mannose, D-glucosamine
3. Hormones of the anterior lobe of the pituitary	
a. Follicle stimulating hormone	D-glucosamine, D-galactose
b. Luteinizing hormone	D-glucosamine
c. Gonadotropic hormone	D-mannose, D-glucosamine, D-galactose
4. Choline esterase	amino sugar
5. Submaxillary mucin	N-acetyl-D-glucosamine, D-mannose

*Mucolipids* (See Section VI).—Compounds which have a fatty residue bound to a carbohydrate residue are known as mucolipids. These substances are sometimes termed lipocarbohydrates, glycolipids, etc., and some may contain amino acid units and phosphorus.

	<i>Identified products of acid hydrolysis of carbohydrate residue</i>
1. Wassermann antigens (Cardiolipin)	D-glucosamine
2. Forssman antigen	D-glucosamine, hexose
3. Bacterial somatic antigens	
a. <i>B. typhosum</i>	hexosamine, D-galactose, D-mannose, D-glucose
b. <i>B. shigae</i>	D-glucosamine, D-galactose, L-rham- nose
c. <i>B. typhimurium</i>	D-glucose, D-galactose, D-mannose
d. <i>Pneumococcus heterophile</i> antigen	D-glucosamine, hexose
e. <i>Brucella melitensis</i>	N-formyl-amino sugar
f. Complex polysaccharides from the tu- bercle bacillus	amino sugar D-arabinose, D-mannose

The above classification and the following description of some of the properties of the carbohydrate constituents of these substances are not exhaustive but are intended primarily to focus attention on them and to illustrate the nature of the problems awaiting study. It is rather remarkable that comparatively few investigations have yet been carried out on their protein constituents, but on attempting structural studies it must be realized that one encounters all the difficulties inherent in protein chemistry with the added complication of the presence of complex carbohydrate molecules.

### III. THE MONOSACCHARIDE UNITS

It will be appropriate to consider first the building units of the carbohydrate residues and it will be immediately apparent that D-mannose and D-galactose with N-acetyl-amino sugars and hexuronic acids play a predominant role. It is proposed, therefore, to discuss briefly some of the more important discoveries connected with the two latter groups and with the carbohydrate sulfates.

### 1. Amino Sugars

On this subject Levene<sup>1</sup> deals adequately with earlier work, much of which was due to his own efforts. Attention has chiefly been focused on the attainment of more refined methods for the characterization and estimation of amino sugars and laterly toward the elucidation of their structure. The whole problem of configurational relationships in the group is closely bound up with the study of anhydro sugars (see S. Peat, this volume, page 37) since such products are produced on deamination of amino sugars, a process which also leads to the added complication of Walden inversion. It is not surprising therefore that the direct mode of attack has not led very far.

Although many more hexosamines than hexoses are theoretically possible only two, namely, D-glucosamine (chitosamine or 2-amino-D-glucose) and chondrosamine (2-amino-D-galactose) have so far been isolated from natural sources. Some synthetic hexosamines have been described by Levene.<sup>1</sup> D-Glucosamine was discovered by Ledderhose<sup>6</sup> in 1878 as the main constituent of chitin and it is only recently that its precise constitution has finally been settled by the Birmingham school (see below). Levene states that "much of the earlier chemical structure was formulated rather by instinct than by experimental evidence"!

The formation of D-glucose phenylosazone from chitosamine showed its relationship to D-glucose and D-mannose and Irvine and Hynd<sup>7</sup> succeeded in preparing both D-mannose and D-glucose from chitosamine.

Attempts to decide whether D-glucosamine is 2-amino-D-glucose or 2-amino-D-mannose have provided a series of fascinating investigations<sup>8-13</sup> the balance of evidence therefrom favoring the D-glucose configuration.

Final proof of the structure of D-glucosamine as 2-amino-D-glucopyranose (I) was furnished as indicated below independently by both chemical<sup>14, 15</sup> and x-ray methods.<sup>16</sup> By the action of ammonia on 2,3-

(6) G. Ledderhose, *Z. physiol. Chem.*, **2**, 213 (1878).

(7) J. C. Irvine and A. Hynd, *J. Chem. Soc.*, **101**, 1128 (1912); **105**, 698 (1914).

(8) P. A. Levene, *J. Biol. Chem.*, **63**, 95 (1925).

(9) M. Bergmann, L. Zervas, H. Rinke and H. Schleich, *Z. physiol. Chem.*, **224**, 33 (1934).

(10) P. Karrer and J. Mayer, *Helv. Chim. Acta*, **20**, 407 (1937).

(11) P. Pfeiffer and W. Christoleit, *Z. physiol. Chem.*, **245**, 197 (1937).

(12) A. G. Widum and A. W. Walker, *J. Infectious Diseases*, **57**, 160 (1935).

(13) C. Neuberg, *Ber.*, **35**, 4009 (1902).

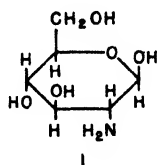
(14) W. O. Cutler, W. N. Haworth and S. Peat, *J. Chem. Soc.*, 1497 (1937).

(15) W. N. Haworth, W. H. G. Lake and S. Peat, *J. Chem. Soc.*, 271 (1939).

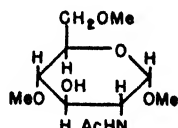
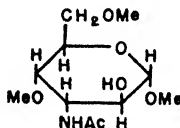
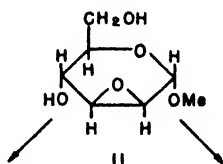
(16) C. G. Cox and G. A. Jeffrey, *Nature*, **143**, 984 (1939).

anhydro-D-mannose a derivative (IV) of 2-amino-D-glucose has been prepared and shown to be identical with the corresponding derivative from D-glucosamine.

Based on the fact that methyl 2,3-anhydro- $\alpha$ -D-mannopyranoside (II) is hydrolyzed<sup>17</sup> by sodium methoxide to give, on subsequent methylation, a mixture of two methyl trimethyl hexosides (methyl 3,4,6-trimethyl- $\alpha$ -D-altropyranoside and methyl 2,4,6-trimethyl- $\alpha$ -D-glucopyranoside) it was anticipated that the action of ammonia on II would follow essentially the same course. This was found to be the case, for with ammonia II yielded compounds which were converted to methyl 4,6-dimethyl-3-acetamido- $\alpha$ -altropyranoside (III) and methyl 4,6-dimethyl-2-acetamido- $\alpha$ -D-glucopyranoside (IV) (10% yield).



D-Glucosamine  
(chitosamine)



Methyl 4,6-dimethyl-  
2-acetamido- $\alpha$ -D-  
glucopyranoside

Thus it is proved that D-glucosamine is related configurationally to D-glucose. This same conclusion was reached independently by Cox and Jeffrey<sup>16</sup> from x-ray analyses of  $\alpha$ -D-glucosamine hydrochloride and hydrobromide. The isomorphism of these substances enabled direct synthetic methods<sup>17a</sup> to be employed without any previous stereochemical assumptions and the atomic positions were determined with high precision.

Both methods of approach moreover proved that a series of methylated derivatives of D-glucosamine described by Cutler, Haworth and Peat,<sup>14</sup> and most useful as reference compounds in this field, were all of the pyranose configuration. Further proof of the pyranose structure of methyl D-glucosaminide and methyl N-acetyl-D-glucosaminide was pro-

(17) W. H. G. Lake and S. Peat, *J. Chem. Soc.*, 1417 (1938); S. Peat and L. F. Wiggins, *ibid.*, 1810 (1938).

(17a) The term "synthetic methods" refers to methods of calculation of electron densities.

vided by Moggridge and Neuberger<sup>18</sup> who measured the energy of activation of these substances as calculated from their hydrolytic constants. It was noted by Peat<sup>19</sup> that the postulation of a betaine structure by Irvine, McNicholl and Hynd<sup>20</sup> to explain the unusual properties of methyl D-glucosaminide hydrochloride is unnecessary.

The high resistance of this glycoside to acid hydrolysis is better explained by the difficulty of approach of the catalyst hydrion to the glycosidic group when a positively charged nitrogen atom is situated on an adjacent carbon atom. In this connection it is of interest to point out that in the two polyglucosamines, namely chitin and the carbohydrate residue of ovomucoid (see below), of which some structural knowledge is available, the amino sugars are linked glycosidically and both polysaccharides are very stable to acid hydrolysis.

Levene has shown that chondrosamine, which can now be prepared readily from the chondroitin sulfate of cartilage<sup>21</sup> is either 2-amino-D-talose or more probably, 2-amino-D-galactose. A constitutional synthesis<sup>21a</sup> of chondrosamine has now been achieved and this shows it to be 2-amino-D-galactose. Levene<sup>22</sup> and Stacey<sup>21</sup> have synthesized a number of acetylated and methylated derivatives of chondrosamine analogous to those available in the D-glucosamine series. Levene<sup>22</sup> has provided evidence of the pyranose structure of methyl N-acetyl-trimethylchondrosaminide—a compound which was shown by Bray, Gregory and Stacey<sup>23</sup> to be derivable from one of the methanolysis products of methylated degraded chondroitin.

Both chondrosamine and D-glucosamine are usually isolated as hydrochlorides but rotational and mutarotational differences between these derivatives are not sufficiently sharp to distinguish between them. The x-ray diffraction pattern is quite distinct for each and the x-ray powder photograph method<sup>21</sup> would appear to be the best for identifying small amounts.

D-Glucosamine in the form of the free base can be obtained in crystalline form by the action of dimethylamine in methanol on the hydrochloride.<sup>24</sup>

*Isolation.*—Useful procedures for the isolation of D-glucosamine have

(18) R. C. S. Moggridge and A. Neuberger, *J. Chem. Soc.*, 745 (1938).

(19) S. Peat, *Ann. Repts. Progress Chem. (Chem. Soc. London)*, 36, 270 (1939).

(20) J. C. Irvine, D. McNicholl and A. Hynd, *J. Chem. Soc.*, 100, 250 (1911).

(21) M. Stacey, *J. Chem. Soc.*, 272 (1944).

(21a) Sybil P. James, F. Smith, M. Stacey and L. F. Wiggins, *Nature*, 156, 368 (1945).

(22) P. A. Levene, *J. Biol. Chem.*, 137, 29 (1941).

(23) H. G. Bray, J. E. Gregory and M. Stacey, *Biochem. J.*, 38, 142 (1944).

(24) R. Breuer, *Ber.*, 31, 2193 (1898).



been devised by Chargaff and Bovarnick<sup>25</sup> and by Neuberger<sup>26</sup> but a better method is that of Jolles and Morgan,<sup>27</sup> by which both D-glucosamine and chondrosamine may be isolated on a micro scale. In the method of Chargaff and Bovarnick the amino sugar is converted by benzyl chloroformate into the sparingly soluble carbobenzyloxy derivative from which the free hexosamine can be regenerated by the hydrogenation process of Bergmann and Zervas.<sup>28</sup> Neuberger used the Schiff base of D-glucosamine with 2,4-dihydroxybenzaldehyde, while Jolles and Morgan, from a study of a number of Schiff bases, concluded that the relatively insoluble azomethine formed from 2-hydroxynaphthaldehyde is eminently suitable. The amino sugar hydrochlorides could readily be regenerated by hydrolysis with 4*N* hydrochloric acid.

Amino sugars can be estimated by the hypiodite method for sugars although a more specific quantitative method has been applied by Elson and Morgan.<sup>29, 30</sup> They made use of a reaction studied by Pauly and Ludwig<sup>31</sup> who had found that when an alkaline solution of an amino sugar was boiled in the presence of ethyl acetoacetate or acetylacetone, the hydrolytic products gave a red color with *p*-dimethylaminobenzaldehyde in hydrochloric acid (the Ehrlich reagent).

## 2. Hexuronic Acids

Hexuronic acids are derived from hexoses in which the primary alcoholic group on C6 is oxidized to a carboxyl group. They occur widely in plant gums, pectins, mucilages, algae, etc., so they may perhaps be more conveniently reviewed when dealing with these groups. Here it is proposed to consider briefly only some established facts concerning their constitution and synthesis in order to illustrate the mode of attack on structural problems in the group. A highly specific test for hexuronic acids consists in boiling them in strong hydrochloric acid solution in the presence of naphthoresorcinol and extracting with benzene the pigment produced. Attempts have been made to adapt the reaction for quantitative estimations, the best method being that of Hanson, Mills and Williams<sup>32</sup> who measure with a photoelectric colorimeter the intensity

(25) E. Chargaff and M. Bovarnick, *J. Biol. Chem.*, **118**, 421 (1937).

(26) A. Neuberger, *Biochem. J.*, **32**, 1435 (1938).

(27) Z. E. Jolles and W. T. J. Morgan, *Biochem. J.*, **34**, 1183 (1940).

(28) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932); *J. Biol. Chem.*, **113**, 341 (1936).

(29) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).

(30) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **28**, 988 (1934).

(31) H. Pauly and C. Ludwig, *Z. physiol. Chem.*, **121**, 176 (1922).

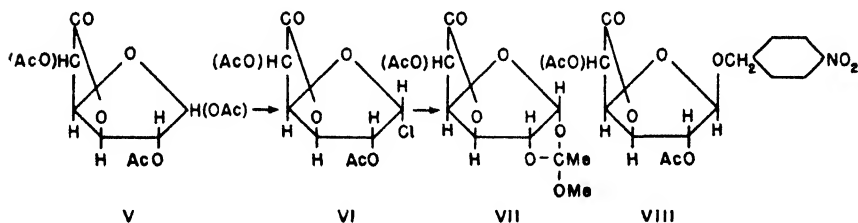
(32) S. W. F. Hanson, C. T. Mills and R. T. Williams, *Biochem. J.*, **38**, 274 (1944).

of the color of an amyl alcoholic extract of the pigment. Other methods of estimation involve the measurement of furfural<sup>33</sup> or of carbon dioxide<sup>34</sup> produced on breaking down the molecule with strong (12%) hydrochloric acid treatment for periods up to twelve hours.

a. *D-Glucuronic Acid*.—This is a substance of high physiological importance since it plays a major role in the detoxication mechanisms of the animal body and it is also of profound significance in determining the immunological specificity of many bacterial polysaccharides and synthetic antigens. When taken by mouth, toxic substances and some drugs, hormones, etc., are in part excreted in the urine in conjugation with *D*-glucuronic acid—usually in the form of *D*-glucuronides. This fact has been adopted by Quick<sup>35</sup> and by Williams<sup>36</sup> as a means of preparing *D*-glucuronic acid. The free acid is most difficult to isolate owing to its ready ease of lactonization to *D*-glucurone (*D*-glucuronolactone).

A number of derivatives of *D*-glucuronic acid have been made by Goebel and Babers<sup>37</sup> with a view to their use in the synthesis of aldobionic acids. With the same object Owen, Peat and Jones<sup>38</sup> prepared both pyranose and furanose derivatives of *D*-glucurone.

On acetylation,<sup>37</sup> *D*-glucurone gives a mixture of  $\alpha$ - and  $\beta$ -triacetyl-*D*-glucuronides (V) which on treatment with acetyl chloride and hydrogen



chloride yields diacetyl-1-chloro-*D*-glucurone (VI) the structure of which, in view of the work<sup>39, 40</sup> described below, should probably now be formulated as 2,5-diacetyl-1-chloro-*D*-glucofuranolactone (VI). When the latter is treated with methyl alcohol and silver carbonate, methyl di-

(33) C. Tollens, *Z. physiol. Chem.*, **64**, 39 (1910); Y. Tanabe, *J. Biochem. (Japan)*, **27**, 251 (1938).

(34) B. Tollens and K. Lefèvre, *Ber.*, **25**, 2569 (1892); K. Freudenberg, H. Gudjons and G. Dumpert, *ibid.*, **74B**, 245 (1941).

(35) A. J. Quick, *J. Biol. Chem.*, **74**, 331 (1927).

(36) R. T. Williams, *Nature*, **143**, 641 (1939).

(37) W. F. Goebel and F. H. Babers, *J. Biol. Chem.*, **100**, 743 (1933); **101**, 173, 707 (1933).

(38) L. N. Owen, S. Peat and W. J. G. Jones, *J. Chem. Soc.*, 339 (1941).

(39) F. Smith, *J. Chem. Soc.*, 584 (1944).

(40) F. Smith, *J. Chem. Soc.*, 1724 (1939).

acetyl-D-glucuronide is formed and it appears to have an orthoacetate structure for the reason that one of the acetyl groups is not hydrolyzed by dilute alkali and the glycosidic group is lost by hydrolysis with extremely dilute acid. This orthoacetate should now be formulated as the fururono-derivative VII. When diacetyl-chloro-D-glucuronolactone is treated with *p*-nitrobenzyl alcohol, a true glycoside, now pictured as VIII, is formed; from this compound, alkali removes both acetyl residues.<sup>41</sup>

The pyranoside structure of methyl 2,3,4-trimethyl- $\beta$ -D-glucopyranoside methyl ester (IX) isolated from the aldobionic acid of gum arabic was established by Challinor, Haworth, and Hirst,<sup>42</sup> while Smith<sup>40</sup> showed that this compound gave a characteristic, easily crystallizable amide (XII). Two crystalline derivatives (XIII and XI) were obtained from D-glucuronolactone by Pryde and Williams<sup>43</sup> on methylation with silver oxide and methyl iodide. These were termed trimethylglucuronolactone and trimethylglucuralone. The latter was shown to be an analog of ascorbic acid, viz., 2,5-dimethyl- $\Delta^4$ -D-glucosaccharolactone methyl ester having the structure XI.<sup>44</sup> Reeves<sup>45</sup> considered that the crystalline trimethylglucuronolactone of Pryde and Williams should be formulated as methyl 2,5-dimethyl-D-glucofururonoside 3,6-lactone (XIII). That it was indeed the  $\alpha$ -form of XIII was conclusively proved by Smith<sup>39</sup> who further suggested that D-glucuronolactone itself may exist free in the furanose form (see also page 103).

By the action of cold acid methyl alcohol on D-glucuronolactone, Owen, Peat and Jones<sup>38</sup> obtained a crystalline compound of empirical formula  $C_7H_{10}O_6$  which was shown to have the structure X, viz., methyl  $\beta$ -D-glucofururonoside 3,6-lactone, by the following series of reactions.

Methylation of X with silver oxide and methyl iodide gave the lactone of methyl 2,5-dimethyl- $\beta$ -D-glucofururonoside (XIII) which was characterized by its conversion to the crystalline amide (XIV). Further methylation of XIII,  $\beta$  form, with methyl sulfate and sodium hydroxide gave methyl 2,3,5-trimethyl- $\beta$ -D-glucofururonoside (XVI) from which by hydrolysis of the glycosidic methyl group and subsequent oxidation with bromine, there was obtained 2,3,5-trimethyl-D-glucosaccharolactone (XVII) characterized by the formation of its crystalline methyl ester.

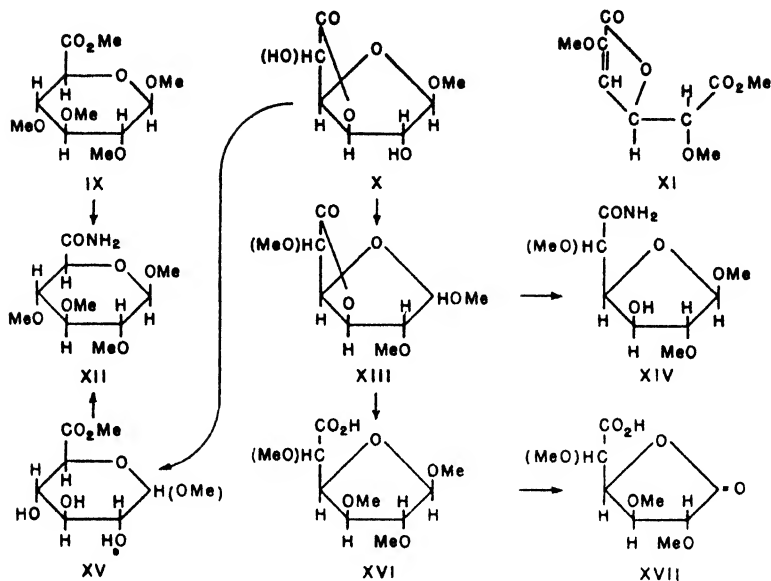
(41) W. F. Goebel and F. H. Babers, *J. Biol. Chem.*, **111**, 347 (1935).

(42) S. W. Challinor, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 265 (1931).

(43) J. Pryde and R. T. Williams, *Biochem. J.*, **27**, 1205 (1933).

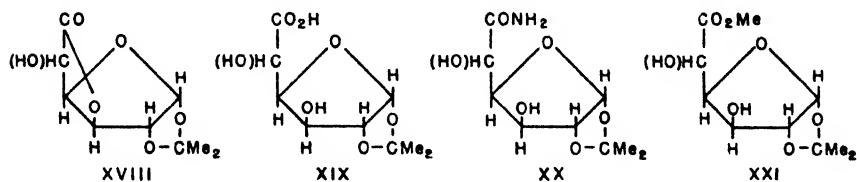
(44) F. Smith, *Chemistry & Industry*, **57**, 450 (1938).

(45) R. E. Reeves, *J. Am. Chem. Soc.*, **62**, 1616 (1940).



Certain anomalous optical and reducing properties of the compound X find a ready explanation in the fact that it possesses two five-membered rings in the molecule, for Smith<sup>44</sup> has shown that such dicyclic systems in the carbohydrate group readily enolize in the presence of alkali. Treatment of X with hot acidic methyl alcohol<sup>38</sup> brings about a ring change from furanose to pyranose and there is produced the methyl ester of methyl D-glucopyruronoside (XV) identified by methylation and conversion to the amide (XII).

Owen, Peat and Jones<sup>38</sup> isolated a valuable derivative (XVIII) of D-glucuronolactone which was characterized as 1,2-isopropylidene-D-



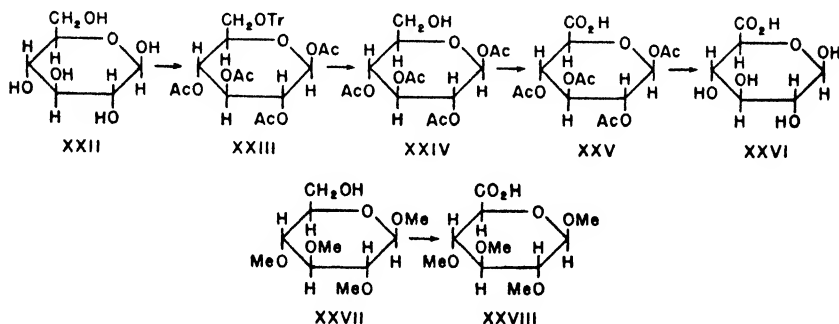
glucofururono-3,6-lactone. This highly crystalline compound is prepared from D-glucuronolactone by condensation with acetone in the presence of sulfuric acid or by lactonizing in a high vacuum the 1,2-isopropylidene-D-glucuronic acid (XIX) of Zervas and Sessler.<sup>46</sup>

Both XVIII and its methyl ester (XXI) readily furnish a crystalline amide (XX) which gives a positive Weerman test for α-hydroxy acids

thereby confirming that the  $-\text{OH}$  group at C5 is unsubstituted and is not engaged in ring formation. Methylation of XVIII by silver oxide and methyl iodide removed the acetone groups giving the unsaturated lactone XI.

*Synthesis of D-glucuronolactone.*—D-Glucuronolactone may be prepared from gum arabic<sup>45a</sup> or by the biological methods of Quick,<sup>35</sup> or Williams.<sup>36</sup> A synthetic method, which is however somewhat cumbersome, is that of Zervas and Sessler<sup>16</sup> which involves the oxidation of 1,2-isopropylidene-3,5-benzylidene-D-glucose with alkaline permanganate and subsequent removal of the acetone and benzylidene groups by acid hydrolysis. A simpler synthetic method has been provided by the reviewer<sup>47</sup> and it appears to be of particular value inasmuch as it is a general one.

The steps involved in the synthesis are as follows: D-glucose (XXII) was converted to 1,2,3,4-tetraacetyl-6-trityl-D-glucose (XXIII) which by



the detritylation method of Helferich and Klein<sup>48</sup> yielded 1,2,3,4-tetraacetyl- $\beta$ -D-glucose (XXIV). Oxidation converted this into 1,2,3,4-tetraacetyl-D-glucuronic acid (XXV), the isolation of which was not essential since by the action of barium hydroxide in aqueous solution, deacetylation with the simultaneous formation of barium D-glucuronate readily took place. Purification of the barium salt and removal of the barium gave D-glucuronic acid which crystallized in the form of the lactone, D-glucurone, in about 20% over-all yield. By a similar procedure methyl 2,3,4-trimethyl- $\beta$ -D-glucuronoside (XXVIII) was synthesized from methyl 2,3,4-trimethyl- $\beta$ -D-glucopyranoside (XXVII).

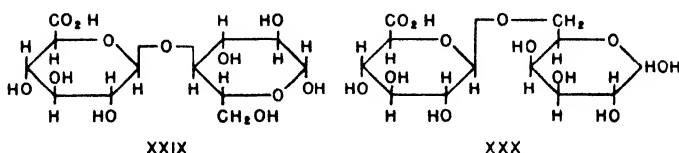
(45a) F. Weinmann, *Ber.*, **62B**, 1637 (1929).

(46) L. Zervas and P. Sessler, *Ber.*, **66B**, 1326 (1933).

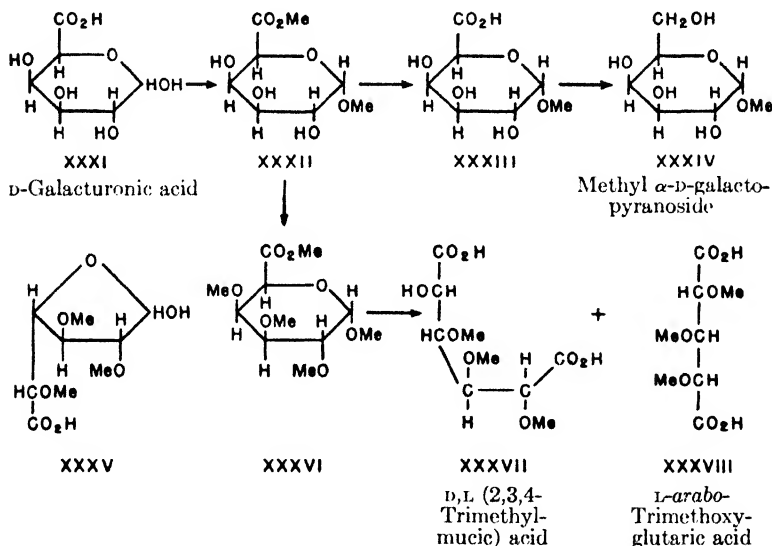
(47) M. Stacey, *J. Chem. Soc.*, 1529 (1939).

(48) B. Helferich and J. Klein, *Ann.*, **450**, 219 (1926).

*b. Aldobionic Acids.*—Among the important aldobionic acids (which contain hexuronic acid) are cellobiuronic acid<sup>49</sup> (XXIX) derived from Type III pneumococcus polysaccharide, and the aldobionic acid<sup>50</sup> (XXX) obtained from gum arabic and also synthesized by Hotchkiss and Goebel.<sup>51</sup>



*c. D-Galacturonic Acid.*—This substance (XXXI) is the sole identified constituent of pectic acid from which it can be obtained by acid or more conveniently, by enzyme hydrolysis (*cf.* Isbell and Frush<sup>52</sup>).



A series of derivatives analogous to those from D-glucuronic acid has been made by Morell and Link.<sup>53</sup> An important compound is the crystalline methyl ester of methyl α-D-galacturonoside (XXXII) which on alkaline hydrolysis yields methyl α-D-galacturonoside (XXXIII). Since

(49) W. F. Goebel, *J. Exptl. Med.*, **64**, 29 (1936).

(50) M. Heidelberger, O. T. Avery and W. F. Goebel, *J. Exptl. Med.*, **49**, 847 (1929).

(51) R. D. Hotchkiss and W. F. Goebel, *J. Biol. Chem.*, **115**, 285 (1936).

(52) H. S. Isbell and Harriet L. Frush, *J. Research Natl. Bur. Standards*, **32**, 77 (1944).

(53) S. Morell and K. P. Link, *J. Biol. Chem.*, **100**, 385 (1933); **104**, 183 (1934).

the rate of acid hydrolysis of the latter substance is of the same order as that of methyl  $\alpha$ -D-galactopyranoside (XXXIV) it is assumed that these galacturonosides have a pyranose structure. Evidence<sup>54</sup> in support of this was obtained from the fact that methyl  $\alpha$ -D-galacturonoside (XXXIII) could be reduced to methyl  $\alpha$ -D-galactopyranoside (XXXIV).

The pyranose structure of D-galacturonic acid, which lactonizes with great difficulty, has been confirmed by Levene and Kreider<sup>55</sup> from a study of the methylated ester of the uronoside. Methyl  $\alpha$ -D-galacturonoside (XXXIII) was methylated with silver oxide and methyl iodide and there was obtained methyl 2,3,4-trimethyl- $\alpha$ -D-galacturonoside methyl ester (XXXVI) which on oxidation was converted to D,L(2,3,4-trimethylmucic) acid (XXXVII) and L-arabo-trimethoxyglutaric acid (XXXVIII).

The methylated compound XXXVI was synthesized by Smith, Stacey and Wilson<sup>56</sup> by the method analogous to that previously used<sup>57</sup> for the synthesis of 2,3,5-trimethyl-D-galacturonic acid (XXXV). Luckett and Smith<sup>57,58</sup> have isolated the methyl ester of methyl 2,3-dimethyl-D-galacturonoside from methylated citrus pectin. D-Galacturonic acid can readily be synthesized from 1,2:3,4-diisopropylidene-D-galactose<sup>59</sup> by oxidation with alkaline permanganate followed by hydrolysis of the acetone groups, or alternatively, by oxidation of 1,2,3,4-tetracetyl-D-galactose with potassium permanganate in glacial acetic acid.<sup>47</sup>

A series of crystalline salts of D-galacturonic acid has recently been prepared by Isbell and Frush.<sup>62</sup> Some of these, e. g., calcium sodium D-galacturonate, are suitable for the separation of D-galacturonic acid from plant materials.

*d. D-Mannuronic Acid.*—This hexuronic acid is the sole identified constituent unit of the sea-weed product alginic acid.<sup>60</sup> 2,3-Dimethyl-D-mannuronic acid (XXXIX) is known and this is oxidized to *i*-dimethoxysuccinic acid (XL) with per-iodic acid followed by bromine.<sup>61</sup>

(54) K. Smoleński and A. Zlotnik, *Bull. intern. acad. polon., Classe sci. math. nat.*, A293 (1934); *C. A.*, 29, 1066 (1935).

(55) P. A. Levene and L. C. Kreider, *J. Biol. Chem.*, 120, 597 (1937).

(56) F. Smith, M. Stacey and P. I. Wilson, *J. Chem. Soc.*, 131 (1944).

(57) Sybil P. Luckett and F. Smith, *J. Chem. Soc.*, 1114 (1940).

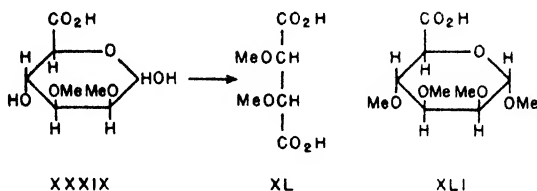
(58) Sybil P. Luckett and F. Smith, *J. Chem. Soc.*, 1106 (1940).

(59) H. Ohle and Gertrud Berend, *Ber.*, 58, 2585 (1925).

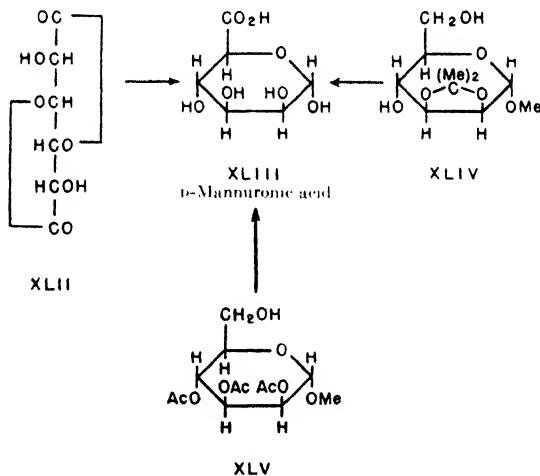
(60) W. L. Nelson and L. H. Cretcher, *J. Am. Chem. Soc.*, 51, 1914 (1929); 52, 2130 (1930); E. L. Hirst, *J. Chem. Soc.*, 70 (1942).

(61) E. L. Hirst, J. K. N. Jones and O. Jones, *J. Chem. Soc.*, 1880 (1939).

The methyl 2,3,4-trimethyl- $\alpha$ -D-mannuronoside (XLI) was synthesized from methyl 2,3,4-trimethyl- $\alpha$ -D-mannoside by the method previously described.<sup>56</sup>



Three routes to the synthesis of D-mannuronic acid (XLIII) are now known. The first<sup>62</sup> was based on the original method of Fischer and Piloty<sup>63</sup> and involved the reduction of D-mannosaccharodilactone (XLII) with sodium amalgam. The second<sup>64</sup> utilized methyl 2,3-isopropylidene- $\alpha$ -D-mannoside (XLIV) which was oxidized at C6 with alkaline potassium permanganate to methyl isopropylidene- $\alpha$ -D-mannuronoside;



this on acid hydrolysis gave D-mannuronic acid. In the third method<sup>65</sup> methyl 2,3,4-triacetyl- $\alpha$ -D-mannoside (XLV) was oxidized with potassium permanganate in glacial acetic acid to give methyl 2,3,4-triacetyl- $\alpha$ -D-mannuronoside which after deacetylation and hydrolysis of the glycosidic methyl group gave  $\beta$ -D-mannuronolactone in good yield.

(62) E. Schoeffel and K. P. Link, *J. Biol. Chem.*, **100**, 40 (1933).

(63) E. Fischer and O. Piloty, *Ber.*, **26**, 521 (1891).

(64) R. G. Ault, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 517 (1935).

(65) M. Stacey and P. I. Wilson, *J. Chem. Soc.*, 587 (1944).



### 3. Sulfuric Esters of Carbohydrates

Levene<sup>1</sup> has outlined the earlier work on the synthesis of three mono-sulfuric esters of D-glucose and stressed the importance of attempts to determine the relative stabilities of sulfuric ester groups on different carbon atoms. The reagents used to introduce sulfate groups directly into carbohydrates or their derivatives were potassium persulfate in alkali, sulfuryl chloride, and chlorosulfonic acid in pyridine, the latter being the most widely applicable. A great advance in dealing with these compounds was the formation of crystalline alkaloidal salts.<sup>66</sup> Typical compounds prepared were the pyridine and sodium salts of tetraacetyl-D-glucose 6- and 1-sulfates, methyl triacetyl- $\beta$ -D-glucoside 6-sulfate, diisopropylidene- $\alpha$ -D-glucose 3-sulfate, isopropylidene-D-glucose 3- and 6-sulfates, etc.

In order to determine the position of the sulfate residues in agar and other polygalactose sulfates, the examination of D-galactose sulfates was begun by Percival and coworkers<sup>67</sup> who first prepared a barium D-galactose 6-sulfate and showed that it differed from a barium D-galactose monosulfate prepared by the direct interaction of D-galactose and chlorosulfonic acid. A comparison of the rates of hydrolysis in acid solution of six hexose and hexoside monosulfates did not provide a means of distinguishing between related hexose sulfates. Alkaline hydrolysis did however show differences and by the action of saturated barium hydroxide on the methyl glycosides of D-glucose and D-galactose sulfates, two methyl anhydrohexosides were produced. It was shown further, that the barium salts of the sulfates of methyl D-galactoside, methyl D-glucoside and methyl D-mannoside all yielded the corresponding methyl 3,6-anhydrohexosides together with some methyl hexosides. The formation of the anhydro rings is of value in locating sulfate groups which, in the above cases must be either on C3 or (more likely) on C6.

In more recent work Percival<sup>68</sup> has shown that on alkaline hydrolysis, 1,2-isopropylidene-D-glucofuranose 6-sulfate gives 1,2-isopropylidene-3,6-anhydro-D-glucofuranose and 1,2-isopropylidene-D-glucofuranose, while 1,2-isopropylidene-D-glucofuranose 3-sulfate (which is very slowly hydrolyzed), gives only 1,2-isopropylidene-D-glucofuranose. From these results and from the examination of other compounds with sulfate groups on C3 or C6, the important conclusion was drawn that with sulfates (unlike *p*-toluenesulfonates) ethylene oxide rings are not necessarily formed on treatment with alkali but 3,6-anhydro rings are formed.

(66) H. Ohle, *Biochem. Z.*, **131**, 601 (1922); T. Soda, *ibid.*, **135**, 621 (1923).

(67) E. G. V. Percival and T. H. Soutar, *J. Chem. Soc.*, 1475 (1940); R. B. Duff and E. G. V. Percival, *ibid.*, 830 (1941).

(68) E. G. V. Percival, *J. Chem. Soc.*, 119 (1945).

## IV. MUCOPOLYSACCHARIDES

1. *Mucopolysaccharides Containing Hexosamine and Uronic Acid*

*a. Hyaluronic Acid.*—The ground substance of connective tissue contains a viscid acidic substance which, like most sulfuric esters of high molecular weight, including mucopolysaccharides, can be differentially stained histologically with toluidine blue. In 1934, Meyer and Palmer<sup>69</sup> described the isolation from the vitreous humor of cattle eyes, of a complex polysaccharide constituted of equimolecular parts of D-glucosamine, D-glucuronic acid and acetic acid, the latter being combined as N-acetyl-D-glucosamine. They named this compound "hyaluronic acid" and it now has assumed great importance since it has been shown to be a substrate for the spreading or diffusing factors. The latter are enzymes, usually mucinases, present in living animal tissues such as testes, in the poisonous secretions of animals and reptiles, and in bacteria; they have a remarkable power of increasing the permeability of connective tissue. An excellent review on these has been presented by Duran-Reynals.<sup>70</sup> *In vivo*, hyaluronic acid, while allowing metabolites to pass through it, has the important function of offering resistance to penetration by foreign matter including agents of infectious disease. It has been isolated from many sources, e. g., aqueous humor,<sup>71</sup> Wharton's jelly of the umbilical cord,<sup>72</sup> synovial fluid,<sup>73</sup> fowl tumor,<sup>74</sup> pleural fluid,<sup>75</sup> pig and rabbit skin,<sup>76</sup> mucoid strains of group A streptococcus,<sup>77</sup> etc. It occurs either free or in salt-like combination with proteins from which it is often difficult to separate. Aqueous solutions of the mucopolysaccharide give a precipitate in the form of a mucin "clot" on adjustment to pH 4 whereas the protein-free polysaccharide gives no precipitate with acid.<sup>78</sup>

The action of spreading factors, e. g., hyaluronidases on hyaluronic acid both *in vivo* and *in vitro* is often quite dramatic, and the effect *in vitro* appears to go in three stages: (a) a separation of the protein residue;

(69) K. Meyer and J. W. Palmer, *J. Biol. Chem.*, **107**, 629 (1934).

(70) F. Duran-Reynals, *Bact. Revs.*, **6**, 197 (1942).

(71) K. Meyer and J. W. Palmer, *Am. J. Ophthalmol.*, **19**, 859 (1936).

(72) K. Meyer, Elizabeth M. Smyth and E. Gallardo, *Am. J. Ophthalmol.*, **21**, 1083 (1938).

(73) K. Meyer, Elizabeth M. Smyth and M. H. Dawson, *J. Biol. Chem.*, **128**, 319 (1939).

(74) E. A. Kabat, *J. Biol. Chem.*, **130**, 143 (1939).

(75) K. Meyer and Eleanor Chaffee, *J. Biol. Chem.*, **133**, 83 (1940).

(76) K. Meyer and Eleanor Chaffee, *J. Biol. Chem.*, **138**, 491 (1941); E. Chain and E. S. Duthie, *Brit. J. Exptl. Path.*, **21**, 324 (1940).

(77) K. Meyer, R. Dubos and Elizabeth M. Smyth, *J. Biol. Chem.*, **118**, 71 (1937).

(78) J. Madinaveitia and M. Stacey, *Biochem. J.*, **38**, 413 (1944).

(b) a rapid depolymerizing action on the polysaccharide with diminution of the viscosity of aqueous solutions; and (c) a partial or complete hydrolytic action with liberation of N-acetyl-D-glucosamine and D-glucuronic acid.

McClean, Rogers and Williams<sup>79, 79a</sup> have devised a most timely use for hyaluronic acid, viz., its use as an early diagnostic test in gas gangrene infections.

The destruction of hyaluronic acid by spreading factors in sperms may play an important role in fertilization processes.<sup>80</sup>

Preparations from various sources show closely similar properties, e. g., nitrogen 3–3.5%, D-glucosamine 30–40%, D-glucuronic acid 40–45%, acetyl 8–12%,  $[\alpha]_D$  –60 to –70° in water.

No structural studies on the chemistry of this important substance have yet been completed.

Meyer<sup>2</sup> considers that the viscosity of aqueous solutions of hyaluronic acid, and the long, doubly refractive fibers obtained by spinning such solutions into a precipitant, are evidence of a long chain type of molecule.

b. *Type I Pneumococcus Polysaccharide*.<sup>80a</sup>—The study of the pneumococci has resulted in their classification into forty different serological types,<sup>81</sup> which differ according to the structure of their capsular polysaccharides.

Type I polysaccharide contains an amino sugar, acetyl residues, and is one of the few bacterial polysaccharides to contain D-galacturonic acid, (another being one described by Hassid<sup>82</sup>). All the pneumococcus polysaccharides listed by Boyd<sup>81</sup> contain acetyl residues, the presence of which in the case of Type I, has a distinct influence on the serological specificity.<sup>83</sup> The structural studies<sup>84</sup> made on the polysaccharide reveal that it may possess a fundamental trisaccharide unit containing two molecules of a uronic acid and one of an acetyl amino sugar, together with an additional acetyl residue.

(79) D. McClean, H. J. Rogers and B. W. Williams, *Lancet*, **244**, 355 (1943).

(79a) D. McClean and H. J. Rogers, *Lancet*, **244**, 707 (1943).

(80) D. McClean and I. W. Rowlands, *Nature*, **150**, 627 (1942); F. Fekete and F. Duran-Reynals, *Proc. Soc. Exptl. Biol. Med.*, **52**, 119 (1942); I. W. Rowlands, *Nature*, **154**, 322 (1944).

(80a) Cf. also page 221.

(81) W. C. Boyd, "Fundamentals of Immunology," Interscience, Inc., New York (1943).

(82) W. Z. Hassid, E. E. Baker and R. M. McCready, *J. Biol. Chem.*, **149**, 303 (1943).

(83) W. F. Goebel, F. H. Babers and O. T. Avery, *J. Exptl. Med.*, **55**, 769 (1932).

(84) O. T. Avery and W. F. Goebel, *J. Exptl. Med.*, **58**, 731 (1933); M. Heidelberger, F. E. Kendall and H. W. Scherp, *ibid.*, **64**, 559 (1936).

c. *Heparin*.<sup>85</sup>—This carbohydrate is probably the natural blood anti-coagulant of the body where it occurs in liver, heart, muscle, etc. It appears to be firmly bound to protein material in tissue since autolytic or proteolytic methods seem to be necessary to liberate it in a soluble form. It was discovered in 1916 by J. McLean<sup>86</sup> in Howell's laboratory and was named heparin by Howell and Holt.<sup>87</sup>

Howell<sup>88</sup> considered it to be a derivative of D-glucuronic acid, but this view was not confirmed by Charles and Scott,<sup>89</sup> who isolated a crystalline barium heparinate from beef lung and detected the presence in it of an amino sugar.

A notable advance was made by Jorpes<sup>90</sup> who showed that heparin could be regarded as a mucoitin sulfuric ester (mucoitin sulfate) which contained much more sulfuric ester than is present in other known mucoitin sulfates. Jorpes emphasized the significance of the sulfur content. He also identified the amino sugar as D-glucosamine and from the amount of CO<sub>2</sub> evolved on strong acid hydrolysis concluded that hexuronic acid residues were present. The hexuronic acid has since been shown to be D-glucuronic acid.<sup>90a</sup>

It was considered that the most active products contained a fundamental tetrasaccharide unit which was esterified with five sulfate groups. Meyer<sup>2</sup> made a careful analysis of crystalline barium acid heparinate (of equivalent weight 750) and his figures were substantiated by the work of Charles and Todd,<sup>91</sup> who showed that lung and liver barium heparinates were identical and who suggested the formula  $(C_{28}H_{39}O_{38}N_2S_5)_2Ba_5 \cdot 24 H_2O$  for a typical preparation. They considered that acetyl residues (1.6–2.9%) were present in the molecule while Reinert and Winterstein<sup>92</sup> found an acetyl content of 10% in a sodium heparinate prepared from a barium salt. The presence of acetyl residues in a carefully purified barium acid heparinate could not be confirmed by Wolfrom and co-workers,<sup>93</sup> who compared the properties of this material with preparations of mucoitin sulfate (from gastric mucin) and chondroitin sulfate from cartilage. The summation of the constituents of both the barium

(85) E. Jorpes, "Heparin," Oxford Univ. Press, London (1939).

(86) J. McLean, *Am. J. Physiol.* **41**, 250 (1916).

(87) W. H. Howell and E. Holt, *Am. J. Physiol.*, **47**, 328 (1918).

(88) W. H. Howell, *Am. J. Physiol.*, **63**, 434 (1923); **71**, 553 (1925).

(89) A. F. Charles and D. A. Scott, *Am. J. Physiol.*, **30**, 1927 (1936).

(90) E. Jorpes, *Biochem. J.*, **36**, 203 (1942).

(90a) M. L. Wolfrom and F. A. H. Rice, *J. Am. Chem. Soc.*, **68**, 532 (1946).

(91) A. F. Charles and A. R. Todd, *Biochem. J.*, **34**, 112 (1940).

(92) M. Reinert and A. Winterstein, *Arch. intern. pharmacodynamie*, **62**, 47 (1939).

(93) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean, *J. Am. Chem. Soc.*, **65**, 2077 (1943).

and sodium salts of heparin was low in comparison with that obtained for chondroitin sulfate and it was anticipated that an unidentified product, possibly a hexose, was present in the molecule. It was considered (*cf.* Charles and Todd, who state that per-iodate has no action on the molecule) that the amino groups were neither free nor acetylated, that all the carboxyl groups are free and that neither carboxyl nor sulfate groups are linked to nitrogen.

On analytical data they give the formula  $C_{24}H_{34}O_{39}N_2S_6Ba_3$  for their preparation, and show that by eliminating the acetyl groups in Charles and Todd's formula and recalculating it they get figures in agreement with their own. Based solely on the analytical figures, the authors present several attractive formulas for heparin. These structures, however, are purely hypothetical and the reviewer deplors the postulation of such formulas as being misleading to non-experts in the field.

It is of interest to note that the crystalline barium heparinate, which is an acid salt, tends to lose its anticoagulant powers very readily and that the authors consider that sulfur content gives no indication of heparin activity.<sup>93a</sup> Some polysaccharide sulfuric esters from marine algae possess anticoagulant activity.

*d. Chondroitin Sulfate*<sup>1</sup>.—This mucopolysaccharide is probably the most readily accessible sulfur-containing carbohydrate which occurs naturally. It forms one of the major constituents of cartilaginous tissue from the trachea, nasal septa, aorta, tendons, sclera, etc. It appears to have first been isolated by Fischer and Boedecker<sup>94</sup> in 1861, and it was prepared in a reasonable state of purity by Krunkenberg<sup>95</sup> in 1884. Most methods of isolation involve a mild saponification process with subsequent removal of liberated protein material in an insoluble form. The average properties of samples of potassium salts are as follows:  $[\alpha]_D - 24^\circ$ ; N, 4%; S, 2.5%; acetyl, 8.5%; potassium, 15%. Meyer and Smyth,<sup>96</sup> however, describe a method of preparation utilizing 10% calcium chloride extraction, which apparently gives a less degraded product (N, 2.95%; S, 5.8%).

The first constitutional studies were made by Schmiedeberg<sup>97</sup> who gave a general idea of the type of structure in chondroitin. Levene<sup>1</sup> considered that the molecule was a tetrasaccharide composed of two glucuronic acid residues conjugated with two units of chondrosamine, esterified with sulfuric acid. On hydrolysis with hydrochloric acid the

(93a) *Cf.* also M. L. Wolfrom and W. H. McNeely, *J. Am. Chem. Soc.*, **67**, 748 (1945).

(94) C. Fischer and C. Boedecker, *Ann.*, **117**, 111 (1861).

(95) C. F. W. Krunkenberg, *Z. Biol.*, **20**, 307 (1884).

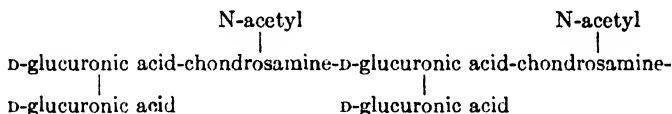
(96) K. Meyer and Elizabeth M. Smyth, *J. Biol. Chem.*, **119**, 507 (1937).

(97) O. Schmiedeberg, *Arch. expil. Path. Pharmacol.*, **28**, 355 (1889).

molecule was degraded to "chondrosine," a disaccharide constituted of one residue of chondrosamine linked to one of D-glucuronic acid—the points of mutual attachment being assumed.

Levene<sup>98</sup> carried out further work on chondrosine, but the structures he presents are only suggestions, since to quote his own words "in this expression the cyclic structures are arbitrary as is also the mode of union of the two components."

The work of Bray, Gregory and Stacey<sup>99</sup> shows that this conception must now be modified. These authors prepared a methylated degraded chondroitin which was sulfate-free and of relatively low molecular weight. From an acid hydrolyzate, derivatives of D-glucuronic acid (including methyl 2,3,4-trimethyl-β-D-glucuronoside) and of chondrosamine (including methyl N-acetyl-3,4,6-trimethyl-chondrosaminide) were isolated and characterized. About one third of the D-glucuronic acid residues were attached glycosidically as terminal residues so that it would appear that the repeating unit is a trisaccharide and that the structure is of the branched chain type as follows:



K. Meyer and his colleagues<sup>100</sup> have conducted careful analytical and preparative studies on compounds in this group. They examined in particular the complexes which chondroitin sulfate formed with proteins and concluded that these were salts involving combination between the  $-\text{COOH}$  and  $-\text{SO}_3\text{H}$  groups of chondroitin sulfate and the  $-\text{NH}_2$  group of proteins.<sup>101</sup> They suggested that hyaline cartilage is a salt of chondroitin sulfate with a gelatin-like protein, and they prepared artificial edestin-chondroitin sulfate fibers having properties closely similar to those of the elastic fibers of connective tissue.

Neuberg and Cahill<sup>102</sup> have carried out fermentation studies on chondroitin sulfate and conclude that it contains D-glucosamine as an additional constituent unit.

*e. Mucoitin Sulfates.*<sup>1</sup>—Levene considered this group to be widely distributed among animal tissues such as gastric mucosa, funis mucin,

(98) P. A. Levene, *J. Biol. Chem.*, **140**, 267 (1941).

(99) H. G. Bray, J. E. Gregory and M. Stacey, *Biochem. J.*, **38**, 142 (1944).

(100) K. Meyer, J. W. Palmer and Elizabeth M. Smyth, *J. Biol. Chem.*, **119**, 501 (1937).

(101) K. Meyer and Elizabeth M. Smyth, quoted in (2).

(102) C. Neuberg and W. Cahill, *Atti accad. Lincei*, (b) **22**, 149 (1935).

vitreous humor, cornea, serum mucoid, ovomucoid, etc., and to differ from chondroitin sulfate only in the fact that its members contain D-glucosamine instead of chondrosamine. These compounds were difficult to free from protein and the ester sulfate residues were relatively labile.

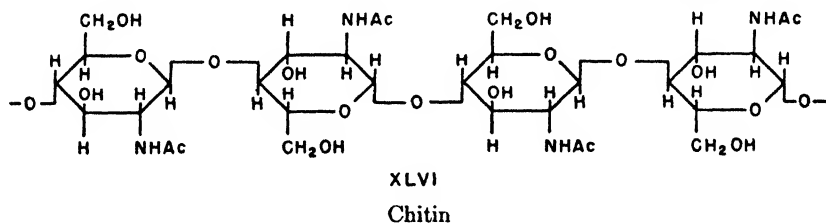
Only two mucoitin sulfates are well authenticated, one from gastric mucin and one from the cornea (hyaluronic acid sulfate).

Following Levene's work, Komarov<sup>103</sup> isolated mucoitin sulfate from the gastric juice of dogs with a gastric fistula and detected in it nitrogen and sulfur, while Meyer and coworkers<sup>104</sup> have described its preparation from commercial hog mucin and have carried out various analytical studies. They obtained samples showing  $[\alpha]_D$  between  $+2$  and  $-8^\circ$  in water, S, 3.1–4.7%; N, 3–4%; D-glucosamine, 30–33%; hexuronic acid, 28–40%, and showed that the fractions had no more than 1% of the anti-coagulant activity of heparin. The function of the mucoitin sulfate is quite unknown.

*f. Hyaluronic Acid Sulfate.*—Meyer and Chaffee<sup>105</sup> showed that this mucopolysaccharide of the cornea was a true derivative of hyaluronic acid, since it could be hydrolyzed enzymically by what is now known as "hyaluronidase."

## 2. Mucopolysaccharides Containing No Hexuronic Acid

*a. Chitin.*—Chitin is the most widely distributed polysaccharide of an amino sugar for it occurs in plants, fungi, animals, and crustacea, as skeletal material and it functions as a highly resistant protective substance. It is remarkably insoluble in all solvents and is highly stable to



acid and alkaline hydrolysis. Its sole constituent unit is N-acetyl-D-glucosamine, several hundred residues of which are linked through the 1,4 positions forming a linear structure, so that chitin may be regarded as 2-acetamido-cellulose (XLVI).

(103) D. R. Webster and S. A. Komarov, *J. Biol. Chem.*, **96**, 133 (1932); S. A. Komarov, *ibid.*, **109**, 177 (1935).

(104) K. Meyer, Elizabeth M. Smyth and J. W. Palmer, *J. Biol. Chem.*, **119**, 73 (1937); K. Meyer and Elizabeth M. Smyth, *ibid.*, **123**, xxxiv (1938).

(105) K. Meyer and Eleanor Chaffee, *Am. J. Ophthalmol.*, **23**, 1320 (1940).

This conclusion<sup>106</sup> has been reached mainly from crystallographic studies on chitin and on acetylchitobiose obtained from chitin by acetolysis.<sup>107</sup> The chitobiose was isolated in the form of its crystalline octa-acetate which could also be obtained<sup>108</sup> by acetylation of the hydrolyzate produced by the action of fuming hydrochloric acid on chitin. A hendecaacetylchitotriose also was obtained by this procedure.

By treatment of chitin with alkali the acetyl groups can be removed and a polyglucosamine formed. This compound, "chitosan," appears to be broken down with nitrous acid even under mild conditions, the sole product being a monosaccharide derivative.

*b. Pneumococcus Polysaccharides.*—The reviewer is of the opinion that all bacterial polysaccharides, whether synthesized by exocellular enzymes, whether forming part of the capsular material, or whether somatic, occur naturally in combination with bound protein or peptide residues. The amount and nature of this polypeptide constituent must determine whether the polysaccharide functions merely as a haptene or whether it can have wider serological properties. There is no doubt that those bacterial polysaccharides isolated by gentle methods show a more marked serological activity. The view has been expressed that in some mucopolysaccharides the protein constituent originates from the synthesizing enzyme and forms a definite part of the structure.<sup>3</sup> The subject of the nature of bacterial polysaccharides (see Mikulaszek<sup>109</sup>) is too wide to be covered in this review and those examples which are included are intended for comparison with the animal products and to draw attention to their biological significance. For a further discussion of bacterial polysaccharides, see page 203.

*Pneumococcus Group (or C) Polysaccharide.*—This substance,  $[\alpha]_D + 61^\circ$  (water), is the polysaccharide constituent of the somatic part of the cell,<sup>110</sup> and is common to all the pneumococcus types. It shows group specificity and is generally similar to the Type IV capsular polysaccharide. Its constituent units are probably N-acetyl-D-glucosamine, a hexose and phosphoric acid. It may be termed the carbohydrate residue of the heterophile antigen (described below) of pneumococcus which was separated by Goebel and his coworkers.

(106) R. O. Herzog, *Naturwissenschaften*, **12**, 958 (1924); H. W. Gonell, *Z. physiol. Chem.*, **152**, 18 (1926); K. H. Meyer and H. Mark, *Ber.*, **61**, 1936 (1928); K. H. Meyer and H. Wehrli, *Helv. Chim. Acta*, **20**, 353 (1937).

(107) M. Bergmann, L. Zervas and E. Silberkreit, *Ber.*, **64**, 2436 (1931).

(108) L. Zechmeister and G. Toth, *Ber.*, **64**, 2028 (1931); **65**, 161 (1932).

(109) E. Mikulaszek, *Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap.*, **17**, 415 (1935).

(110) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **53**, 625 (1931).



*Type IV Pneumococcus Specific Polysaccharide.*—This capsular material<sup>110</sup> ( $[\alpha]_D + 33^\circ$ , water) has been hydrolyzed and shown to contain units of D-glucose and N-acetyl-hexosamine. Its structure has not yet been studied.

*Type XIV<sup>111</sup> Pneumococcus Specific Polysaccharide* ( $[\alpha]_D + 12^\circ$ , water).—An antigenic relationship of theoretical and practical interest between this substance and human erythrocytes has been observed and serves to explain some of the fatal reactions obtained with the use of Type XIV pneumococcus antisera. The latter contain antibodies for human erythrocytes of Types A, B, AB and O, whereas antisera from all other types do not. These antibodies are directed against the capsular polysaccharides of the organisms for they can be removed from the serum by adsorption with Type XIV pneumococcus or its capsular polysaccharide.<sup>112</sup> Since the antibodies are also directed against polysaccharides contained in human erythrocytes, a structural relationship must exist between the Type XIV capsular polysaccharide and an erythrocyte carbohydrate. This was clearly proved by Goebel, Beeson and Hoagland,<sup>111</sup> who demonstrated the close relationship between the Type XIV material and a purified Blood Group A factor from peptone (described below). Further detailed work on these relationships is clearly desirable.

*c. Blood Group Specific Substances* (see Wiener<sup>113</sup>).—These may be divided into two classes—the group specific substances proper which constitute the “agglutinin” portion of erythrocytes, and other substances which display specific activity but which can be isolated from a wider variety of sources such as gastric mucin and saliva.

These specific substances or “blood group factors” are detected by their property of inhibiting agglutinin reactions between human erythrocytes and heterologous human sera. This is the so-called “isoagglutinin test” which can detect the factors in dilutions of more than 1 part in 10 million. An even more sensitive but less specific test is their power to inhibit hemolysis of sheep's erythrocytes by the serum of rabbits immunized with certain human corpuscles.

Little is known of the chemical nature of the group specific substances proper for they are extremely difficult to isolate. Active alcoholic extracts have been obtained from erythrocytes by various workers<sup>113</sup> and in some instances these extracts were shown to contain carbohydrates.

(111) W. F. Goebel, P. B. Beeson and C. L. Hoagland, *J. Biol. Chem.*, **129**, 455 (1939).

(112) A. J. Weil and E. Sherman, *J. Immunol.*, **36**, 139 (1939).

(113) A. S. Wiener, “Blood Groups and Transfusion,” C. C. Thomas, Baltimore (1943); F. Schiff and L. Adelsberger, *Zentr. Bakt. Parasitenk. I Orig.*, **93**, 172 (1924).

Kossjakow<sup>114</sup> claims to have isolated a polysaccharide in this way, by a relatively simple procedure, but the reviewer and coworkers<sup>115</sup> have been unable to obtain active material by this method. It does appear that the specific substances contain carbohydrate, lipid, and possibly polypeptide constituents similar perhaps to the Forsman antigen (see below) and that the linkages between these constituents are very fragile.

The preparation of "blood group factors" is a relatively simple matter, and some progress has been made with regard to elucidating their chemical nature. Highly active Group "A" factors have been prepared from hog pepsin,<sup>116</sup> human urine,<sup>117</sup> horse saliva,<sup>118</sup> hog mucin,<sup>119</sup> commercial peptone,<sup>120</sup> human saliva<sup>115, 121</sup> and gastric juice,<sup>115</sup> pseudomucinous cystic fluid,<sup>122</sup> etc. Group "O" and Group "B" factors have been isolated<sup>115, 122-124</sup> from human gastric juice and saliva, while active substances have been shown<sup>125</sup> to be present in other tissue fluids of so-called "secreters," such as in tears, sweat, semen, etc.

In all cases where the chemical properties of these preparations have been studied the active material appears to be predominantly carbohydrate in nature, containing in the case of the "A" factor 50-70% of reducing sugar, 5.7% nitrogen and giving by colorimetric estimations 25-30% of glucosamine on hydrolysis.

Freudenberg<sup>117</sup> and his colleagues obtained evidence of the presence of D-galactose and N-acetyl-D-glucosamine in their preparations and showed the high biological significance of the presence in them of the acetyl groups.

The B- and O-factors made by Witebsky and Klendshoj<sup>123</sup> contained less nitrogen (1.5-1.6% in the case of the "B" factor and 2.8% in the case of the "O" factor) and the "B" factor gave 75% of reducing sugars on hydrolysis as compared with 39.8% reducing sugar given by the "O" factor. One doubts whether these analyses are of real significance. All

(114) P. N. Kossjakow and G. P. Tubulew, *Z. Immunitäts.*, **98**, 26 (1940); **99**, 221 (1941).

(115) H. G. Bray, H. Henry and M. Stacey, *Proc. Biochem. Soc.*, **38**, xxi (1944).

(116) B. Brahn, F. Schiff and F. Weinmann, *Klin. Wochschr.*, **11**, 1592 (1932); K. Landsteiner and M. W. Chase, *J. Exptl. Med.*, **63**, 851 (1936).

(117) K. Freudenberg and H. Eichel, *Ann.*, **518**, 97 (1935).

(118) K. Landsteiner, *J. Exptl. Med.*, **63**, 185 (1936).

(119) W. T. J. Morgan and H. K. King, *Biochem. J.*, **37**, 640 (1943).

(120) W. F. Goebel, *J. Exptl. Med.*, **68**, 221 (1938).

(121) K. Landsteiner and R. A. Harte, *J. Biol. Chem.*, **140**, 673 (1941).

(122) H. K. King and W. T. J. Morgan, *Proc. Biochem. Soc.*, **38**, x (1944).

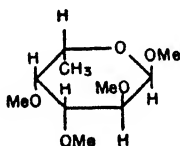
(123) E. Witebsky and N. C. Klendshoj, *J. Exptl. Med.*, **73**, 655 (1941).

(124) K. I. Yosida, *Z. ges. exptl. Med.*, **63**, 331 (1928).

(125) H. G. Bray and M. Stacey, *Biochem. J.*, in press (1946).

active factors contain a small but significant amino acid content, and Landsteiner and Harte<sup>121</sup> have suggested that these amino acids are an integral part of the structure and have a role in determining the serological specificity of the complex. This view appears to be correct for it can be shown that removal of amino acid constituents is always accompanied by a loss of specificity.<sup>115, 119-120</sup> It has also been shown<sup>123-124</sup> that there is no simple relationship between nitrogen, or reducing sugar contents and biological activity, or between optical activity and group specificity. Little work of a detailed chemical nature has been carried out on these substances, but in the course of a study by the methylation process,<sup>115</sup> of the A substance prepared by mild saponification of commercial pepsin, it has been shown that the carbohydrate fraction of the complex contains units of D-mannose, D-galactose, N-acetyl-D-glucosamine, and L-fucose.

The latter was isolated in the form of fully methylated methyl fucoside, i. e., methyl 2,3,4-trimethyl- $\alpha$ -L-fucoside (XLVII), a compound previously obtained from methylated gum tragacanth.<sup>125</sup> The recognition of this particular derivative indicates that the L-fucose residues in part at least must constitute end groups linked glycosidically to the rest of the molecule.<sup>3</sup> The same compound<sup>125</sup> was isolated by methylation and



XLVII

hydrolysis of the Group A factor prepared by Morgan from hog gastric mucin<sup>119</sup> by extraction with phenol. This method gives a highly potent substance, aqueous solutions of which show considerable viscosity. The native "A" factor is readily degraded by heat treatment and there is produced a substance which shows a lower viscosity in water and which has a reduced power to inhibit isoagglutination. This degraded material unexpectedly showed a slight increase in its power to inhibit A-specific hemolysis.

An interesting practical application of these factors has been initiated by Morgan,<sup>127, 128</sup> who prepared an artificial antigen with Group A specificity by combining the Blood Group A factor from hog mucin with a

(126) Sybil P. James and F. Smith, *Proc. Biochem. Soc.*, **38**, xxi (1944).

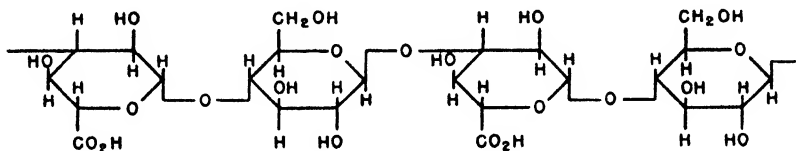
(127) W. T. J. Morgan, *Chemistry & Industry*, **60**, 722 (1941).

(128) W. T. J. Morgan, *Brit. J. Exptl. Path.*, **24**, 41 (1943).

polypeptide-like component of the "O" somatic antigen of *Bacterium dysenteriae* Shiga. Antisera against the Group A complex agglutinated Group A erythrocytes in dilutions up to one part in twelve thousand, but did not agglutinate the group B erythrocytes appreciably more than did normal sera.

### 3. Mucopolysaccharides Containing No Hexosamine

A wide range of bacterial polysaccharides<sup>128a</sup> is known containing up to 1% nitrogen which is not present as an amino sugar constituent. The nitrogenous constituent is considered as being a "vestigial remain," and, as already stated,<sup>3</sup> the reviewer considers that it is a protein constituent in combination with the polysaccharide and originates from the polysaccharide-synthesizing enzymes. The high degree of cross specificity<sup>129</sup> exhibited by these highly colloidal substances has been clearly shown to be due to the close structural relationship between their aldobionic acid constituents. The structure of the Type III pneumococcus capsular substance has been worked out by Reeves and Goebel, who show that it has a linear structure constituted of a chain of cellobiuronic acid residues joined through the 1,3 positions<sup>130</sup> as in XLVIII.



XLVIII

The polysaccharides of Type VIII pneumococcus,<sup>131</sup> Friedlander's bacillus,<sup>132</sup> *Rhizobia*<sup>133</sup> and *Azotobacter* evidently have closely related structures and they contain a high proportion of D-glucose residues. Cross reactions indicated that oxidized cellulose has affinities with this group.<sup>134</sup>

The mechanism of the conversion of cellulose by the *Cytophagae*<sup>136</sup> into viscous mucoproteins of high molecular weight forms an unexplored subject of great interest.

(128a) Cf. page 205.

(129) J. R. Marrack, "Chemistry of Antigens and Antibodies," Med. Research Council, London (1938).

(130) R. E. Reeves and W. F. Goebel, *J. Biol. Chem.*, **139**, 511 (1941).

(131) W. F. Goebel, *J. Biol. Chem.*, **110**, 391 (1935).

(132) W. F. Goebel, *J. Biol. Chem.*, **74**, 619 (1927).

(133) Elsa Schlüchterer and M. Stacey, *Biochem. J.*, **38**, 154 (1944).

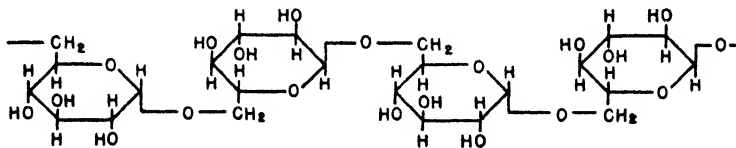
(134) M. Heidelberger and Gladys L. Hobby, *Proc. Natl. Acad. Sci.*, **28**, 516 (1942).

(136) E. Walker and F. L. Warren, *Biochem. J.*, **32**, 31 (1938).

#### 4. Mucopolysaccharides Without Hexosamine or Uronic Acid Residues

In this group we place mainly the neutral bacterial "slimes" and reserve carbohydrates. They are better defined products than those previously dealt with and as such may of course be regarded as true polysaccharides. Invariably, however, saponification methods are required to rid them of protein residues and to make them water-soluble. The more soluble mold polysaccharides appear to lose their protein constituents by autolytic processes during the longer periods required for mold metabolism. Mold "slime" production can, however, readily be demonstrated on a solid medium. It is proposed here to give briefly some of the types of structure known in the group.

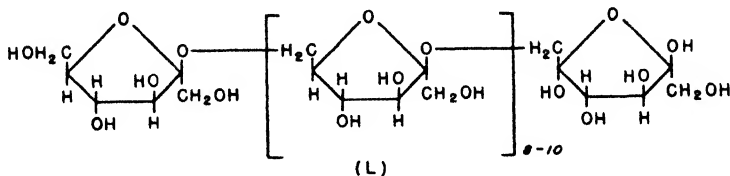
*a. Bacterial Dextrans (Polyglucoses).*<sup>137</sup>—These constitute the slimes which cause "ropy sugar." They are formed from sucrose by various species of *Leuconostoc* bacteria. The following is a typical structure:



XLIX

In XLIX the chains may be long or short, linear or branched, and the D-glucose residues are mainly joined through the 1,6-position. These substances are discussed in more detail in a succeeding chapter (page 209).

*b. Bacterial Levans (Polyfructoses).*<sup>138</sup>—These are formed by a wide variety of aerobic organisms including plant pathogens. The following repeating unit is common to all and it is identical with that from the



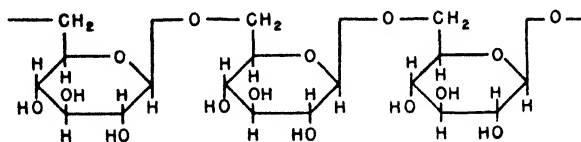
plant levans. It is a contiguous chain of twelve D-fructofuranose units joined by 2,6-linkages (L). These substances are discussed in more detail in a succeeding chapter (page 225).

(137) H. R. L. Tarr and H. Hibbert, *Can. J. Research*, 5, 414 (1931); F. L. Fowler, I. K. Buckland, F. Brauns and H. Hibbert, *ibid.*, 15, 486 (1937); S. Peat, Elsa Schlüchterer and M. Stacey, *J. Chem. Soc.*, 581 (1939); M. Stacey and W. D. Daker, *ibid.*, 584 (1939).

(138) R. R. Lyne, S. Peat and M. Stacey, *J. Chem. Soc.*, 237 (1940).

c. *Yeast Mannan*<sup>139</sup> (see p. 224).—This is one of the reserve carbohydrates of yeast. A mold product mannocarolose<sup>140</sup> has a closely related structure.

d. *Luteic Acid*<sup>141</sup> (see p. 224).—This acidic mold polysaccharide is unusual for the reason that it contains units each of which is composed of two molecules of D-glucose and one molecule of malonic acid, the attachment of the latter being by an ester link through only one of the carboxyl groups, the other remaining free. On mild acid or alkaline hydrolysis the malonic acid residues are readily split off leaving a neutral polysaccharide "luteose," the constitution of which is as follows:



LiI

Luteose

e. *Galactogen from Snail Mucin*.<sup>142</sup>—The mucin from the body of snails is mentioned by Levene<sup>1</sup> and it has excited interest for some considerable time. The polysaccharide from the albumin glands of the snail *Helix pomatia* appears to have been described first by Hammarsten in 1885<sup>143</sup> under the name "animal sinistrin." It was later studied by May<sup>144</sup> who obtained an identical product from the eggs of the snail and concluded that it was a polygalactose which he termed "galactogen." Baldwin and Bell<sup>142</sup> prepared galactogen from dried albumin glands by digesting them with 30% potassium hydroxide and they submitted it to careful study by the methylation procedure. Hydrolysis of the methylated product gave approximately equimolecular proportions of 2,3,4,6-tetramethyl-D-galactose and 2,4-dimethyl-D-galactose. On this evidence they postulate two alternative means of formulating the structure of

(139) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 833 (1941).

(140) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, 29, 612 (1935).

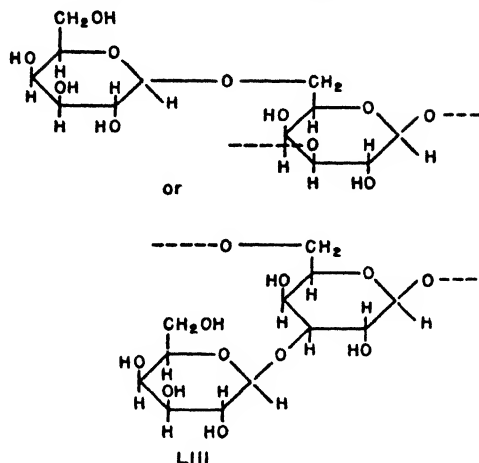
(141) H. Raistrick and M. Rintoul, *Trans. Roy. Soc. (London)*, B220, 225, (1931); C. G. Anderson, W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, 33, 272 (1939).

(142) E. Baldwin and D. J. Bell, *J. Chem. Soc.*, 1461 (1938); 125 (1941).

(143) O. Hammarsten, *Arch. ges. Physiol. (Pflügers)*, 36, 373 (1885).

(144) F. May, *Z. Biol.*, 92, 321, 325 (1932); 95, 277, 401, 606, 614 (1934).

galactogen, either of which is of a novel type:



In a further investigation it was shown that in the repeating unit there are four terminal residues, one of which is L-galactose.

## V. MUCOPROTEINS

### 1. Ovomuroid

In this group, ovomucoid has been subjected to a detailed study for it is a more readily accessible substance. The presence of polysaccharide material in egg white has been known for some considerable time and it has been the subject of numerous studies (for literature see Neuberger,<sup>145</sup> Meyer<sup>2</sup>). It would appear that all the egg proteins contain a carbohydrate residue, since it has been shown that the carbohydrate content of crystalline egg albumin remains constant<sup>145</sup> on recrystallization, and it is impossible to separate the carbohydrate material from the protein by ultrafiltration or denaturation methods. A polysaccharide can, however, be separated by removal of the protein portion by tryptic digestion followed by purification involving acetylation and deacetylation. It has a molecular weight of about 1200 and contains four molecules of D-mannose and two of D-glucosamine, together with an unidentified nitrogenous constituent. The carbohydrate residue in ovomucoid<sup>146</sup> differs from this polysaccharide by being more stable to acid hydrolysis, in optical properties and in its possessing acetic acid and D-galactose units in addition to D-glucosamine and D-mannose.

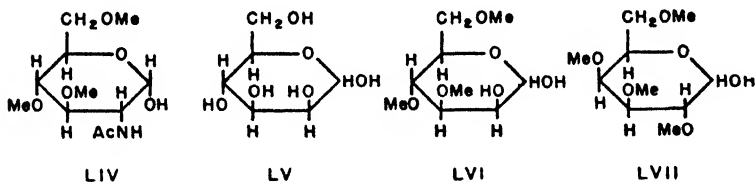
Ovomucoid is the name given to the material precipitated by alcohol

(145) A. Neuberger, *Biochem. J.*, **32**, 143 (1938).

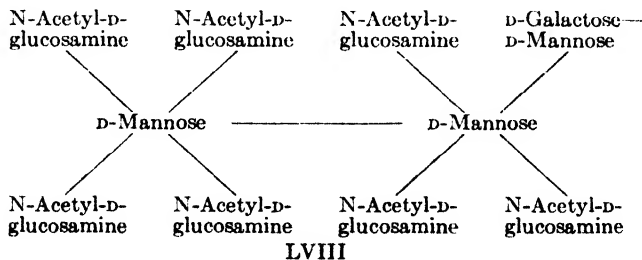
(146) M. Stacey and J. M. Wooley, *J. Chem. Soc.*, 184 (1940); 550 (1942).

from a solution in which albumins and globulins have been removed by heat coagulation. It appears to contain tryptophane, tyrosine, arginine, histidine, D-mannose, N-acetyl-D-glucosamine and D-galactose. Meyer<sup>2</sup> prefers to distinguish between an ovomucoid- $\alpha$  and an ovomucoid- $\beta$ , the latter being a rather ill-defined substance which separates out as a viscous product on dilution of egg white with water and which becomes remarkably insoluble on being kept. Most workers (*cf.* however, Sørensen and Haugaard<sup>147</sup>) have failed to detect the presence of D-galactose in ovomucoid, terming the carbohydrate a "glucosamine-dimannoside," and differences between various preparations well illustrate how the nature of a protein-carbohydrate complex can vary with the mode of isolation, particularly in the hands of different workers.

It was found possible to saponify ovomucoid with sodium hydroxide and to methylate simultaneously with methyl sulfate, the carbohydrate residue thereby forming an N-acetyl methyl derivative.<sup>146</sup> Hydrolysis of this material yielded N-acetyl-3,4,6-trimethyl-D-glucosamine (7 moles) (LIV), D-mannopyranose (2 moles) (LV), 3,4,6-trimethyl-D-mannopyranose (1 mole) (LVI), and tetramethyl-D-galactopyranose (1 mole) (LVII).



From the identification of these products it was possible to give a means of portraying the type of structure (LVIII) present in the carbohydrate molecule. In this, by glycosidic attachment, seven N-acetyl-D-glucosamine units and one D-galactose unit radiate from a central core of three D-mannose units. It is not yet clear by what linkages the D-mannose units are mutually attached, or to which D-mannose the D-galactose residue is linked.



(147) Margrethe Sørensen and G. Haugaard, *Biochem. Z.*, 260, 247 (1933); *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, 22, 487 (1933).



It is considered that LVIII does not represent a repeating unit but rather that it depicts the whole molecule as being that of a hendecasaccharide having a type of structure which appears to be unique in carbohydrate chemistry. The general properties of the carbohydrate residue support the contention that it is not a polysaccharide of high molecular size. In regard to the structure of ovomucoid itself, of which the carbohydrate forms 20%, it would appear that the peptide constituents are mainly attached to the N-acetyl-D-glucosamine terminal residues. Moreover, since they can be split therefrom by saponification methods, they may be attached by ester linkages through the 3-, 4- and 6-hydroxyl groups of the amino sugars. Support for the glycosidic mode of attachment of the N-acetyl-D-glucosamine constituents was forthcoming from the work of Levene<sup>148</sup> who isolated from a carbohydrate group of egg proteins a D-glucosamine-D-mannose disaccharide in 40% yield. The observation that reduction of this disaccharide converted the D-mannose moiety into D-mannitol led to its formulation as D-glucosaminido-D-mannose.

## 2. Serum Mucoproteins

The question of the presence of carbohydrate residues as essential bound constituents of proteins such as albumins and globulins has long been the subject of considerable research and is still controversial. Information on the structure of these carbohydrate residues is very scanty and is mainly derived from colorimetric measurements as indicated below. The earlier literature on the subject has been surveyed by Rimington.<sup>149</sup> Investigations had shown that both serum albumin and globulin gave a Molisch reaction indicative of the presence of carbohydrate, but the complex attached polysaccharide was not isolated from each until 1929.<sup>150</sup> D-Glucosamine and D-mannose were identified as constituent units and the work of Sorensen and Haugaard<sup>147</sup> made it likely that D-galactose was also a constituent.

Sørensen and Haugaard<sup>147</sup> showed that by a suitable fractionation of crystalline albumin there could be obtained a series of fractions which, although crystalline, varied widely in their properties, particularly in regard to their carbohydrate contents. The most soluble fraction contained about fifty times as much carbohydrate as the least soluble. In most of these studies the carbohydrates were estimated by modification of the orcinol colorimetric method of Tillmans and Philipi<sup>151</sup> which could

(148) P. A. Levene, *J. Biol. Chem.*, **140**, 279 (1941).

(149) C. Rimington, *Ergeb. Physiol. expil. Pharmacol.*, **35**, 712 (1933).

(150) C. Rimington, *Biochem. J.*, **23**, 430 (1929).

(151) J. Tillmans and K. Philipi, *Biochem. Z.*, **215**, 36 (1929).

be applied to most proteins. With careful controls the method gives valuable comparative results but is of doubtful value as a quantitative method since there may be some destruction of the liberated parts of the molecule before the rest is hydrolyzed.

Hewitt<sup>152</sup> has carried out extensive studies on the "bound" carbohydrates in proteins and has obtained from serum albumin essentially carbohydrate-free and carbohydrate-rich components. He regards purified crystalline albumin (crystalbumin) as being devoid of a carbohydrate residue. From crude crystalbumin he separated two mucoproteins, globoglycoid and seroglycoid. The former was prepared from the mother liquors after crystallization of crystalbumin or from globulin precipitated by 45–55% saturated ammonium sulfate solution, while the latter was separated from serum after successive removal of globulin and crystalbumin by ammonium sulfate. Hewitt considered that these various proteins were simple admixtures. He further observed that seroglycoid resembled the seromucoid of earlier literature, but produced evidence that the two were not identical. This seromucoid was discovered in 1892 by Freund<sup>153</sup> who gave it the name "animal gum," but failed to detect nitrogen in it. It was renamed seromucoid by Zanetti<sup>154</sup> from its analogy to ovomucoid. This mucoid, which was obtained from serum after heat coagulation of the albumins and globulins, was studied by Bywaters<sup>155</sup> who showed that it contained 25% of a carbohydrate, part of which was D-glucosamine (detected by the formation of its penta-benzoate). A preparation made by Ozaki<sup>156</sup> gave only 13.4% of reducing sugars after acid hydrolysis. A complete review of the problem was undertaken by Rimington<sup>157</sup> who also (with Van den Ende<sup>158</sup>) provided methods for preparing crystalbumin, globoglycoid (crystalline), seroglycoid and seromucoid from horse serum. Seromucoid contained 10.7% of a polysaccharide consisting of N-acetyl-D-glucosamine, D-mannose and D-galactose in equimolecular proportions, along with tyrosine (3%), tryptophan (1%) and cystine (2.3%). In a number of protein fractions examined the ratio hexose/D-glucosamine was always found to be 2/1. It appeared that, as shown by chemical and immunological methods, crystalbumin and globoglycoid are essentially identical and differ from

(152) L. F. Hewitt, *Biochem. J.*, **30**, 2229 (1936); **31**, 360 (1937); **32**, 260 (1938); **33**, 1496 (1939).

(153) H. Freund, *Z. physiol. Chem.*, **6**, 345 (1892).

(154) C. U. Zanetti, *Ann. chim. farm.*, **26**, 529 (1897).

(155) H. W. Bywaters, *Biochem. J.*, **15**, 322 (1921).

(156) G. Ozaki, *J. Biochem. (Japan)*, **24**, 215 (1936).

(157) C. Rimington, *Biochem. J.*, **34**, 931 (1940).

(158) C. Rimington and M. Van den Ende, *Biochem. J.*, **34**, 941 (1940).

seroglycoid. The latter, although closely related to seromucoid, was not identical with it in immunological properties.

Since antibodies occur in the globulin fraction of serum one predicts that the investigation of the fine structure of serum mucoproteins will become a problem of urgent immunological significance. It is doubtful however whether the carbohydrate group of mucoproteins can influence their immunological specificity.<sup>159</sup>

The physiological significance of mucoproteins in serum is unknown but it is tempting to speculate that they may simply be intermediate stages in the synthesis of albumins and globulins. However, it will be seen below that mucoproteins with hormone properties can be isolated from serum of pregnant mares, and that the enzyme choline esterase, is probably a serum mucoprotein.

### 3. Gonadotropic Substances

It seems reasonably well established that gonadotropic substances are mucoproteins containing hexose and hexosamine units. Thus Fischer and Ertel<sup>160</sup> stated that a preparation made from the anterior pituitary lobe agreed closely in properties with ovomucoid and urinary mucoid. Meyer and Kurzok<sup>161</sup> recognized that a highly active follicle stimulating hormone (a mucoprotein) contained amino sugar and acetyl residues, while Gurin and coworkers<sup>162</sup> estimated that their preparation contained hexose and hexosamine in the ratio 2/1, and further concluded from the orcinol method that the hexose was galactose (12%).

Evans and coworkers<sup>163</sup> examined the products in the 40% alcoholic extract of sheep pituitary and reported that the interstitial cell stimulating or luteinizing hormone (I. C. S. H.) contains carbohydrate (3.6 to 5.4%; glucosamine, 3.8%) and the follicle stimulating hormone (F. S. H.) also contains carbohydrate (10.3 to 13.1%; glucosamine, 8%). They regard the carbohydrate content (glucosamine in particular) as being a measure of the F. S. H. activity.

Gurin<sup>164</sup> has tabulated data from an examination of gonadotropic substances derived from several sources. The luteinizing hormone and the F. S. H. from the pituitary are stated to contain D-mannose and

(159) A. Neuberger and M. E. Youill, *Biochem. J.*, **34**, 109 (1940).

(160) F. G. Fischer and L. Ertel, *Z. physiol. Chem.*, **83**, 202 (1931).

(161) K. Meyer and R. Kurzok, "The Endocrines in Obstetrics," Williams and Wilkins, Baltimore, p. 115 (1937).

(162) S. Gurin, C. Bachman and D. W. Wilson, *Science*, **89**, 62 (1939).

(163) H. M. Evans, H. Fraenkel-Conrat, M. E. Simpson and C. H. Li, *Science*, **89**, 249 (1939); H. M. Evans, M. E. Simpson and C. H. Li, *Endocrinology*, **27**, 803 (1940).

(164) S. Gurin, *Proc. Soc. Exptl. Biol. Med.*, **49**, 48 (1942).

hexosamine in equimolecular proportions. The gonadotropic hormone from the serum of pregnant mares was shown to contain D-galactose (or possibly D-mannose and D-glucosamine in equimolecular amounts) and hexosamine in the ratio of 2/1, while the chorionic gonadotropic hormone ("prolan") from human pregnancy urine contained D-galactose and hexosamine in these same proportions.

Rimington and Rowlands<sup>165</sup> have isolated an extremely active preparation from the serum of pregnant mares. This material was obtained in a high degree of purity and was shown to contain hexose and hexosamine in the molar ratio of 2/1. In the fractionation it appeared that, at first, the hexose content decreases as the potency is raised, but later this trend is reversed. It was shown that hexose content could not be taken as indication of gonadotropic activity. The hexose/hexosamine ratio was 2/1 as in normal serum mucoproteins, so that this gonadotropic hormone may be a modified serum mucoprotein.

The thyreotropic hormone<sup>166</sup> from pituitary extracts appeared to be an entity separate from the luteinizing hormone and contained nitrogen, 13%; carbohydrate, 3.5%; and D-glucosamine, 2.5%.

Further work both on the protein and carbohydrate constituents is awaited with interest, for it has been shown by Evans and Hauschildt<sup>167</sup> that the activity of the hormone of pregnant mares' serum is a function of the whole molecule, inasmuch as it is destroyed both by proteolytic and carbohydrate-splitting enzymes.

#### 4. Choline Esterase

By a careful fractionation of normal horse serum, involving as an essential part of the process a separation of closely related substances by the Schütz<sup>168</sup> foam technique, Bader, Schütz and Stacey<sup>169</sup> obtained a crystalline mucoprotein with high choline esterase activity. This appears to be the first mucoprotein obtained without the use of heat or alcohol, and while it is not yet claimed that the crystalline material is indeed the enzyme itself, arguments are advanced to show that the enzymic activity is closely bound up with mucoprotein structure.

(165) C. Rimington and I. W. Rowlands, *Biochem. J.*, **35**, 736 (1941); *Nature*, **152**, 355 (1943); *Biochem. J.*, **38**, 54 (1944).

(166) H. Jensen and S. Talkdorf, *Proc. Soc. Exptl. Biol. Med.*, **42**, 466 (1939); J. Fraenkel-Conrat, H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans, *J. Biol. Chem.*, **135**, 199, 235 (1940).

(167) J. S. Evans and J. D. Hauschildt, *J. Biol. Chem.*, **145**, 345 (1942).

(168) F. Schütz, *Nature*, **139**, 629 (1937).

(169) R. Bader, F. Schütz and M. Stacey, *Nature*, **154**, 183 (1944).

### 5. Submaxillary Mucin

The mucin of the submaxillary gland appears to contain two mucoproteins.<sup>170</sup> One is a D-glucosamine-di-D-mannose complex similar to that in the serum proteins, while the other is claimed to be a crystalline substance  $C_{14}H_{26}O_{11}N$ , containing D-glucosamine, two acetyl residues and a polyhydroxy acid which is not a uronic acid. It appears to be a type of sulfate-free chondroitin.

Grassmann and Schleich<sup>171</sup> have shown by Sørensen's method that ox-skin collagen contains equimolecular amounts of D-glucosamine and D-galactose, and a complex with the same proportions of these constituents has been reported<sup>172</sup> in the protein of the ovalbumic glands of *Rana esculenta*. The mucoproteins of urine are as yet unexplored except insofar as they are known to contain small amounts of certain of the blood group factors.<sup>116</sup>

## VI. MUCOLIPIDS

In this group are placed certain compounds of high practical immunological significance as particularly exemplified in the case of the Wassermann substance, the reaction of which is employed in the diagnosis of syphilis and as a guide to the progress of therapy. This is the most widely used of all serological tests. It is not proposed to include cerebro-sides in this group until it is known more precisely whether the carbohydrate groups in these are polysaccharides or simply glycosides. While there is an extensive literature dealing with the immunological properties of mucolipids, chemical knowledge is very scanty. It does appear, however, that the biological specificity of members of the group is determined by the presence in them of a carbohydrate haptene.

The Wassermann substance<sup>173</sup> is prepared by extracting various animal organs, particularly beef heart, with alcohol, and its lipidal nature was early recognized. Pangborn<sup>174</sup> described the preparation of a new phospholipid termed "cardiolipin" from beef heart and claimed that it was the essential constituent of the Wassermann substance. On hydrolysis it gave a fatty acid and a phosphorylated polysaccharide. In a later communication<sup>175</sup> however, the carbohydrate constituent is stated

(170) G. Blix, C. Oldfeldt and O. Karlberg, *Z. physiol. Chem.*, **234**, iii (1935).

(171) W. Grassmann and H. Schleich, *Biochem. Z.*, **277**, 320 (1935).

(172) P. Schultz and M. Becker, *Biochem. Z.*, **280**, 217 (1935).

(173) A. J. Weil, *Bact. Revs.*, **5**, 293 (1943).

(174) Mary C. Pangborn, *J. Biol. Chem.*, **143**, 247 (1942); **153**, 343 (1944).

(175) L. Buchbinder, *Arch. Path.*, **19**, 841 (1935); J. Forssman in W. Kolle, R. Krause and P. Uhlenhuth, "Handbuch der Pathogenen Micro-organismen," Jena, 3rd ed., **3**, p. 469 (1930).

to be due to an impurity. Weil<sup>173</sup> has ably reviewed the literature on the Wassermann and related antigens.

The Forssman antigens<sup>176</sup> may be regarded as heat-stable substances, which, when injected into rabbits, can evoke sheep-cell hemolysins.

Unlike most serological reactions which are amazingly specific the interactions of these so-called "F" or heterophile antigens show a high degree of cross-specificity, which can now be traced to the presence in them of chemically similar constituents. The "F" antigens are widely distributed in nature, being present in the tissues of mammals, birds, fishes, yeasts, and bacteria. They have been classified into four groups.<sup>176</sup> Studies on the "F" antigens as they occur in animal tissues suggest that they are carbohydrate-lipo-protein<sup>177</sup> complexes and Brunius<sup>178</sup> considers that the carbohydrate portion contains D-glucosamine units.

More knowledge is available on the heterophile antigens from bacteria, particularly those in the gram-negative group. Thus with the "O" somatic antigen from *Bacterium dysenteriae* Shiga,<sup>179, 180</sup> it was possible to demonstrate that on injection into rabbits it would evoke the formation of heterophile antibodies and moreover that only the polysaccharide-peptide part of the mucolipid was necessary for this antibody production.

It was shown simultaneously by Boivin and coworkers<sup>181</sup> and by Raistrick and Topley<sup>182</sup> that the antigens of certain gram-negative organisms can be isolated in a relatively pure condition and unchanged in their specific immunological properties. The latter authors provided good evidence that though their antigens were essentially free from intact protein they were highly toxic and efficient immunizing agents. Work along similar lines has been carried out by Topley and coworkers<sup>183</sup>, Mesrobianu,<sup>184</sup> Morgan,<sup>185</sup> Miles and Pirie,<sup>186</sup> and by Goebel and co-

(176) F. P. Gay, "Agents of Disease and Host Resistance," C. C. Thomas, Baltimore, p. 340 (1935).

(177) K. Landsteiner and P. A. Levene, *J. Immunol.*, **14**, 81 (1927).

(178) F. E. Brunius, "Chemical Studies on the True Forssman Haptene, etc.," Stockholm (1936).

(179) K. H. Meyer and W. T. J. Morgan, *Brit. J. Exptl. Path.*, **16**, 309, 476 (1936).

(180) W. T. J. Morgan and S. M. Partridge, *Biochem. J.*, **34**, 169 (1940); **35**, 1140 (1941).

(181) A. Boivin and Lydia Mesrobianu, *Compt. rend. soc. biol.*, **115**, 304, 309 (1934).

(182) H. Raistrick and W. W. C. Topley, *Brit. J. Exptl. Path.*, **15**, 113 (1934).

(183) W. W. C. Topley, H. Raistrick, J. Wilson, M. Stacey, S. W. Challinor and R. O. J. Clark, *Lancet*, **232**, 252 (1937).

(184) Lydia Mesrobianu, "Les Antigens Glucido-Lipidiques des Bacteries," Paris (1936).

(185) W. T. J. Morgan, *Biochem. J.*, **31**, 2003 (1937).

(186) A. H. Miles and N. W. Pirie, *Brit. J. Exptl. Path.*, **20**, 278 (1939).

workers.<sup>187</sup> The various methods which have been used have involved extraction of cells with trichloroacetic acid, trypsin, diethylene glycol, phenol, guanidine, etc., followed by precipitation of the antigens by alcohol or acetone. All methods appear to give closely related complexes of high molecular weight. The *B. typhimurium* somatic antigen contains a complex constituted of four components: a specific polysaccharide, a polypeptide, an acetyl polysaccharide, and a phospholipid. These constituents, which are produced on gentle hydrolysis,<sup>188</sup> have been examined in some detail by Freeman.<sup>189</sup> The polysaccharide which readily combined with protein was considered to be built up from units of D-glucose (19%), D-mannose (21.5%) and D-galactose (19%). The *B. typhosum* antigen<sup>178, 183, 188</sup> is a similar complex containing a polysaccharide (50–60%),  $[\alpha]_D + 114^\circ$  in water, an insoluble polypeptide (16%), a soluble nitrogenous constituent (10–20%), and a lipid component (3–4%). The polysaccharide yielded D-galactose, D-mannose, and D-glucose on hydrolysis. It appears that the *B. typhosum* polysaccharide contains D-glucosamine also as a constituent unit.<sup>190</sup>

*B. dysenteriae* Shiga<sup>178</sup> contains a closely related complex, the polysaccharide component of which yielded on hydrolysis D-galactose, L-rhamnose and N-acetyl-D-glucosamine. The toxicity of these antigenic preparations appears to be largely due to the undegraded complex. Examination of the individual components revealed many interesting facts. Thus with *B. shigae* the specific polysaccharide failed to induce any demonstrable antibodies, while the polypeptide component engendered homologous precipitins of low titer but no agglutinins against *B. shigae*. A "reconstituted" polysaccharide-polypeptide complex, however (made by mixing the two substances in formamide), induced the formation of specific immune serum of high titer. A polysaccharide-lipid complex was only weakly antigenic. In an extensive study of *Brucella melitensis*, Miles and Pirie<sup>186</sup> extracted with an aqueous phenol-chloroform mixture an antigenic substance of high molecular weight. It gave on mild hydrolysis a mixture of lipids and a N-formylamino sugar.

The lipid constituents of the acid-fast bacilli, particularly the tubercle bacillus, have been extensively studied and there is good evidence that carbohydrate-lipid complexes play an important part in the sero-

(187) W. F. Goebel, T. Shedlovsky, G. I. Lavin and H. H. Adams, *J. Biol. Chem.*, **148**, 1 (1943).

(188) M. Stacey, S. W. Challinor and H. Raistrick, *Rept. Proc. Int. Cong. Microbiol.*, 356 (1937); G. G. Freeman, S. W. Challinor, J. Wilson, *Biochem. J.*, **34**, 307 (1940); G. G. Freeman and T. H. Anderson, *Biochem. J.*, **35**, 564 (1941).

(189) G. G. Freeman, *Biochem. J.*, **37**, 601 (1943).

(190) P. I. Wilson, Thesis, University of Birmingham (1943).

logical reactions of these organisms. Thus Heidelberger and Menzel<sup>191</sup> have prepared from the somatic part of the tubercle bacillus several specific polysaccharides which were shown to be constituted of D-mannose, D-arabinose and D-galactose units. In some cases the polysaccharides were esterified with palmitic acid. The presence of D-glucosamine in tubercle polysaccharides has been reported by Anderson.<sup>192</sup> It has also been suggested that the wax from the tubercle bacillus may contain fatty esters of carbohydrates.<sup>193</sup> An interesting finding of great importance is that the lipid fractions extracted by chloroform, alcohol, etc., can give rise to the typical cellular reactions<sup>194</sup> associated with tubercle formation. A resumption of these researches is awaited with interest, for it is expected that they will eventually lead to the replacement of our present-day crude vaccines for prophylactic treatment of diseases by chemical entities of known composition.

(191) M. Heidelberger and A. E. O. Menzel, *J. Biol. Chem.*, **118**, 79 (1937); cf. E. Chargaff and W. Schaefer, *Ann. inst. Pasteur*, **54**, 708 (1935).

(192) R. J. Anderson, *Am. Rev. Tuberc.*, **22**, 664 (1930).

(193) R. J. Anderson, *Chem. Revs.*, **29**, 225 (1941).

(194) Florence R. Sabin and A. L. Joyner, *J. Exptl. Med.*, **68**, 853 (1938).





# BACTERIAL POLYSACCHARIDES

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## I. INTRODUCTION

Bacterial polysaccharides were first recognized nearly one hundred years ago, but information about their structures and properties has been obtained almost entirely during the last fifteen or twenty years. Combined with protein, these polysaccharides are responsible for the immunizing powers of many bacteria, and the structure of the polysaccharide determines the exact or specific immunological response to these organisms.

Polysaccharide formation may be endocellular, exocellular or capsular. The polysaccharide is usually a normal metabolic product, frequently a major product. Isolation and purification of a bacterial polysaccharide generally involve continued precipitations from a buffered solution, together with electrodialysis or ultrafiltration.

For bacterial polysaccharides removal of the last traces of nitrogen by denaturation is frequently very difficult, and may involve degradation of the polysaccharide.<sup>1</sup> The exact nature of this nitrogenous constituent is not clear. It may arise from the nucleoprotein of the enzymes responsible for polysaccharide synthesis. Short chains of D-glucose residues may be joined progressively by the enzymic nucleoprotein to form the macromolecular bacterial polysaccharide.<sup>2</sup> However, there are indications<sup>3</sup> that this small amount of nitrogen may not be responsible for the immunological reactions of the polysaccharide.

Most early publications on bacterial polysaccharides were concerned with impure products and poorly-described organisms. Many more recent papers are of limited value also, due to low yields, lack of characterization of products and arbitrary interpretations of data. Low yields of methylated polysaccharides may be due to degradation of the bacterial polysaccharide during methylation, or to degradation of the hydrolytic products of the methylated polysaccharide (to form methyl levulinate, etc.<sup>5,6</sup>). The great importance of: (a) complete methylation of polysaccharide products prior to structural determination by hydrolysis; and (b) quantitative identification of the hydrolytic products, has been emphasized previously.<sup>6</sup> Other difficulties in end group analysis have been discussed recently.<sup>7</sup>

One of the recent fundamental advances in carbohydrate chemistry has been enzymic synthesis *in vitro*; this has now been realized with dextran<sup>8</sup> and with levan.<sup>9</sup> However, since phosphate sugars are not involved in the enzymic syntheses of these two bacterial polysaccharides, it is obvious that phosphorylation is just one process for the natural synthesis of polysaccharides.

Bacterial cellulose, dextran and many other bacterial polysaccharides are composed entirely of D-glucose units; the levans are condensation polymers of D-fructose. Dextrans from different species of *Leuconostoc*

- (1) M. Stacey and F. R. Youd, *Biochem. J.*, **32**, 1943 (1938).
- (2) M. Stacey, *Chemistry & Industry*, **62**, 110 (1943).
- (3) T. H. Evans, W. L. Hawkins and H. Hibbert, *J. Exptl. Med.*, **74**, 511 (1941).
- (5) I. Levi, W. L. Hawkins and H. Hibbert, *J. Am. Chem. Soc.*, **64**, 1959 (1942).
- (6) S. Peat, E. Schlüchterer and M. Stacey, *J. Chem. Soc.*, 581 (1939).
- (7) D. J. Bell, *J. Chem. Soc.*, 473 (1944).
- (8) E. J. Hehre and J. Y. Sugg, *J. Exptl. Med.*, **75**, 339 (1942).
- (9) S. Hestrin, S. Avineri-Shapiro and M. Aschner, *Biochem. J.*, **37**, 450 (1943).

have different chemical structures, whereas levans synthesized by widely different organisms apparently have similar structures. The polysaccharides of pathogenic bacteria are usually composed of more than one carbohydrate; D-galactose, D-mannose, pentoses, amino sugars and acetylated sugars are possible constituents, in addition to D-glucose.

This review deals with bacterial and related polysaccharides, such as those of molds and yeasts. The bacteriological nomenclature is that of Bergey<sup>10</sup>; non-systematic nomenclature is indicated by (?).

## II. BACTERIAL CELLULOSE

### 1. Early Studies

In 1886, Brown<sup>11</sup> discovered an organism which formed extremely tough membranes when cultivated in suitable nutrient solutions containing carbohydrates such as D-fructose, D-mannitol or D-glucose; ethanol, sucrose or starch did not support membrane formation by this organism which Brown called *Bacterium xylinum*(?) (*Acetobacter xylinum*). The membranes were readily soluble in cuprammonium hydroxide solution and yielded a dextrorotatory sugar upon acid hydrolysis. These properties and the results of combustion analysis led him to believe that the membrane was cellulose.

C. A. Browne<sup>12</sup> studied the cellulosic material obtained by fermentation of Louisiana sugar cane juice. His results substantiated those of A. J. Brown, and he further showed that since the nitrogen content of the membranes was only 0.2%, chitin could only be present with the cellulose membrane in traces, if at all.

Other early workers reported the formation of cellulose by various other organisms,<sup>13</sup> e.g., *Acetobacter pasteurianum*, *Acetobacter rancens*, *Sarcina ventriculi* and *Bacterium xylinoides*.

Tarr and Hibbert<sup>13</sup> published the first detailed study of the formation of bacterial cellulose. A systematic series of experiments, conducted with a view to obtaining a culture medium which did not support visible growth of *A. xylinum* until a suitable source of carbon was added, indicated that a solution (pH 5.0) containing 0.1% asparagine, 0.5% potassium dihydrogen phosphate, 0.1% sodium chloride and 0.5% ethanol satisfied these requirements. Maximum polysaccharide formation oc-

(10) D. H. Bergey, "Manual of Determinative Bacteriology," Williams and Wilkins Co., Baltimore, Maryland, 5th ed. (1939).

(11) A. J. Brown, *J. Chem. Soc.*, 49, 172, 432 (1886); 51, 643 (1887).

(12) C. A. Browne, Jr., *J. Am. Chem. Soc.*, 28, 453 (1906).

(13) H. L. A. Tarr and H. Hibbert, *Can. J. Research*, 4, 372 (1931).

curred at 30° ten days after inoculation, and the addition of ethanol to the culture medium caused a very marked increase in yield. It was shown that polysaccharide formation occurred in this culture medium only after the addition of hexose sugars (5–10% concentration), their anhydrides, D-mannitol or glycerol, which presumably could be converted readily into hexoses by *A. xylinum*. D-Fructose was the most suitable hexose substrate, followed by D-glucose, D-galactose and D-mannose; higher yields of bacterial cellulose were obtained with D-fructose polysaccharides such as levan and inulin for substrates than with D-glucose polysaccharides such as starch and dextrin. D-Glucose is partially converted to D-gluconic acid by *A. xylinum* and the yields of cellulose from D-glucose are therefore less than those obtained with D-fructose which gives rise to only small amounts of acid. *A. xylinum* oxidizes D-mannitol to D-fructose, and glycerol to dihydroxyacetone which may then be converted to D-fructose. Disaccharides and polysaccharides which may require preliminary hydrolysis gave relatively low yields of bacterial cellulose, whereas pentoses, ethylene glycols, D,L-( $\alpha$ -methylglycerol) and methyl  $\alpha$ -D-glucoside did not give rise to polysaccharide formation.

The synthesis of cellulose by *A. xylinum* from various polyalcohols<sup>14</sup> is accompanied by the formation of carbon dioxide, formic acid, non-volatile acids, ketoses and sometimes ethanol. The much greater variety of substrates suitable for cellulose synthesis, as compared with the small number for dextran or levan, may account for the widespread natural occurrence of cellulose.

## 2. Chemical Structure

Hibbert and Barsha<sup>15</sup> conducted the first thorough chemical study of bacterial cellulose. Production of the cellulose membrane could be regarded as due entirely to the carbohydrate employed, since *A. xylinum* would not grow in the nutrient medium<sup>13</sup> in the absence of the carbohydrate. The bacterial cellulose was made up of a nearly infinite number of closely-compacted membranes each of which might absorb as much as one hundred times its own weight of water. Repeated boiling with water yielded the purified product free from reducing sugars and nitrogen, but containing 0.3% ash. The yield was relatively low, *e. g.*, thirty-five liters of culture medium containing 1750 g. of D-glucose yielded 72 g. of pure dried membrane or approximately 4.1% of the D-glucose used.

(14) Yvonne Khouvine, *Bull. soc. chim. biol.*, 18, 1325 (1936).

(15) H. Hibbert and J. Barsha, *Can. J. Research*, 5, 580 (1931).

Owing to the compactness of the dried membrane, bacterial cellulose showed even greater resistance than cellulose to the action of the various chemical reagents used in the determination of its structure.<sup>16</sup> Acetylation of the product with acetic anhydride and acetic acid using sulfuryl chloride as catalyst gave a quantitative yield of a triacetate having the same properties as the triacetate prepared from cotton cellulose. Deacetylation of the triacetate with alcoholic sodium hydroxide yielded the starting material unchanged. This regenerated cellulose was quantitatively hydrolyzed to D-glucose by means of zinc chloride in hydrochloric acid. Acetolysis of bacterial cellulose with a mixture of acetic anhydride and sulfuric acid yielded cellobiose octaacetate. Treatment of the cellulose triacetate with methanol containing hydrogen chloride yielded 94% of methyl  $\alpha$ - and  $\beta$ -D-glucosides. Simultaneous deacetylation and methylation (twice, by means of dimethyl sulfate and sodium hydroxide) of partially acetylated bacterial cellulose, followed by two methylations with methyl iodide in the presence of silver oxide, gave an 85% yield of trimethylcellulose ( $\text{OCH}_3$ , 44.0%). The latter, on hydrolysis with methanol containing hydrogen chloride, yielded methyl 2,3,6-trimethyl-D-glucosides (92% yield) which in turn were converted into crystalline 2,3,6-trimethyl-D-glucose (83% yield). Since the same products were formed in similar yields from natural cellulose, it was concluded that bacterial cellulose produced from D-glucose is identical with natural cellulose.

Barsha and Hibbert<sup>16</sup> also demonstrated by means of methylation, acetylation, acetolysis and hydrolysis experiments that the membranes synthesized by the action of *A. xylinum* on D-fructose and on glycerol were chemically identical with cotton cellulose.

Schmidt and coworkers<sup>17</sup> showed by conductometric titrations that bacterial cellulose resembled all undegraded celluloses in its content of 0.28% of carboxyl groups.

### 3. X-ray and Electron Microscope Investigations

Further confirmation of the identity of bacterial and plant celluloses has been obtained by x-ray studies. From dried membranes prepared by the action of *A. xylinum* on sucrose, Eggert and Luft<sup>18</sup> obtained x-ray diagrams similar to those of cotton cellulose. Hibbert and Barsha<sup>15</sup> showed that a chloroform solution of the triacetate of cellulose

(16) J. Barsha and H. Hibbert, *Can. J. Research*, **10**, 170 (1934).

(17) E. Schmidt, R. Schnegg, W. Jandebaur, Margarete Hecker and W. Simson, *Ber.*, **68B**, 542 (1935).

(18) J. Eggert and F. Luft, *Z. physik. Chem., Abt. B*, **7**, 468 (1930).

(prepared by the action of *A. xylinum* on D-glucose) yielded a cellulose acetate fiber on dry spinning identical with cotton cellulose acetate fibers. On hydrolysis of the acetate fibers with alcoholic sodium hydroxide, the regenerated cellulose gave an x-ray diagram which had the typical diffraction pattern of natural cellulose. It was further shown by x-ray investigation<sup>16</sup> that purified membranes prepared by the action of *A. xylinum* on D-fructose, glycerol, sucrose, D-galactose and D-mannitol consisted of well-developed crystallites of cellulose which had a preferential orientation; membranes dried under tension showed a greater degree of preferred orientation.

Franz and Schiebold<sup>19</sup> studied bacterial cellulose by means of the electron microscope and x-rays. An Ardenne vibrator was used to separate (partially or completely) the organisms from the membranes; seven micrographs (magnification 10,000–40,000 times) illustrate the results. In some cases bacteria were connected to single fibers of cellulose about 100 Å thick, 5000 Å wide and up to 40 microns in length. There were indications that the organisms produced single threads about 200 Å wide, and that these became “woven” together. Prolonged electronic irradiation decomposed the membranes. The x-ray and electron diffraction studies indicated a “ring fiber” (“*Ringfaser*”) structure in the dried and unstretched membrane. Stretching of the moist membranes produced parallel orientation.

#### 4. Industrial Importance

Since bacterial cellulose from all suitable carbohydrate substrates is identical with natural cellulose, its industrial importance<sup>20</sup> is obvious. Relatively large amounts of bacterial cellulose were produced in Germany during the first World War. More recently products similar to parchment, mercerized cotton, cellulose nitrate,<sup>21</sup> acetate<sup>15</sup> and viscose rayons have been produced from bacterial cellulose.

From a theoretical standpoint, further study of the formation of bacterial cellulose might yield some information regarding the mechanism of plant synthesis.

### III. DEXTRANS

#### 1. The History of Dextran

The term “dextran” has been applied to carbohydrate slimes originating from sugar sirups, fermenting vegetables and dairy products.

(19) E. Franz and E. Schiebold, *J. makromol. Chem.*, **1**, 4 (1943).

(20) G. A. Brossa, *Ann. Microbiol.*, **2**, 77, 150 (1942); *C. A.*, **38**, 5635 (1944).

(21) Yvonne Khouvine, G. Champetier and R. Sutra, *Compt. rend.*, **194**, 208 (1932).



Attention was first directed toward the dextrans about the middle of the last century as these slimes were at times a serious problem in the wine and beet sugar industries. In the latter, jelly-like masses blocked the filters and interfered with the refining processes by retarding crystallization. Dextran, at various times, was regarded as a protoplasmic constituent of the beet cell,<sup>22</sup> a product of sucrose fermentation, and as cellulose produced by fermentation.<sup>23</sup> The empirical formula,  $(C_6H_{10}O_5)_n$ , for dextran was obtained<sup>22, 23</sup> at an early date (about 1875); on hydrolysis, the substance was found to yield only D-glucose.<sup>12, 22-24</sup> A tribenzoate and a triacetate of dextran were prepared by Däumichen.<sup>24</sup>

The early chemical and bacteriological work on dextran has been reviewed elsewhere<sup>25-27</sup> in detail. Early bacteriological studies of the dextrans were complicated by non-systematic nomenclature and incomplete description of the organisms. Considerable differences have been noted in the cultural characteristics and morphology<sup>26</sup> of the various strains of *Leuconostoc*. It is now known that the dextrans are produced by the action on sucrose of certain strains of chain-forming cocci, classified by Hucker and Pederson<sup>26</sup> as the Family *Coccaceae*, Tribe *Streptococceae*, Genus *Leuconostoc*, Species *L. mesenteroides* and *L. dextranicum*. *Leuconostoc mesenteroides* (Cienkowski) van Tieghem ferments pentoses (either D-arabinose or D-xylose) and sucrose, whereas *Leuconostoc dextranicum* Beijerinck ferments sucrose but not pentoses. Dextran is not produced by the third species of *Leuconostoc*, *L. citrovorum*, and this species ferments neither sucrose nor pentoses. In general, those species of *Leuconostoc* which ferment sucrose produce in addition to dextran (ca. 25% crude yield on the basis of sucrose) approximately 30% (*levo*)-lactic acid, 5% acetic acid, 10% ethanol and 10% D-mannitol.<sup>26</sup>

Tarr and Hibbert<sup>27</sup> determined the optimal conditions for the preparation of dextran. Sucrose was found to be the only suitable carbohydrate substrate, although some strains produced a small amount of dextran from D-glucose. The formation of dextran from D-glucose had been observed occasionally by earlier workers, but such formation is of somewhat transient character, occurring only when the cultures are very active with respect to the formation of polysaccharides from sucrose.<sup>27</sup> The nutrient solution (pH 8.0) used by Tarr and Hibbert<sup>27</sup> for dextran

(22) C. Scheibler, *Z. Ver. deut. Zucker-Ind.*, **19**, 472 (1869); **24**, 309 (1874).

(23) E. Durin, *Compt. rend.*, **83**, 128 (1876).

(24) P. Däumichen, *Z. Ver. deut. Zucker-Ind.*, **40**, 701 (1890).

(25) F. Lafar, "Handbuch der Technischen Mykologie," Bd. 1, 2, G. Fischer, Jena (1908).

(26) G. J. Hucker and C. S. Pederson, *N. Y. Agr. Expt. Sta., Tech. Bull.* **167**, 3 (1930).

(27) H. L. A. Tarr and H. Hibbert, *Can. J. Research*, **5**, 414 (1931).

formation consisted of sucrose (10%), peptone (0.1%), potassium chloride (0.1%) and disodium hydrogen phosphate (0.2%), and incubation was allowed to proceed for ten days at room temperature. The crude dextran was isolated from the concentrated culture by precipitation with methanol and was purified by electrodialysis.

More recent preparations of dextran have shown the need of supplementing the medium of Tarr and Hibbert,<sup>27</sup> especially if the organisms have been cultured for any great periods of time on artificial media. The formation of dextran from sucrose also may be a transient character, and *Leuconostoc* organisms may require activation by several passages through suitable media before dextran formation is appreciable, or the power to produce dextran is regained. This activation may merely re-establish an adequate concentration of growth factors. It has been pointed out by many workers that certain accessory substances are necessary for the promotion of growth of *Leuconostoc*,<sup>26</sup> e. g., yeast extract, peptone, tomato extract, purine and pyrimidine bases.<sup>28</sup> Gaines and Stahly<sup>29</sup> have recently reported that thiamine, calcium pantothenate, nicotinic acid, pyridoxine and probably biotin are essential for growth of *L. mesenteroides*.

The following substances have been added to culture media to increase the yields of dextran: raw beet sugar or molasses,<sup>30</sup> commercial maple sirup,<sup>1</sup> yeast extract,<sup>31</sup> magnesium and ammonium sulfates,<sup>32</sup> tomato juice,<sup>3,31</sup> calcium carbonate,<sup>3</sup> and a water extract of waste sugar-refining charcoal (probably containing materials related to the vitamin B complex).<sup>33</sup>

Dextran is probably identical with the capsular carbohydrate of *Leuconostoc*, but capsules<sup>3</sup> have not always been observed, even with *Leuconostoc* organisms which actively produced dextran.<sup>27,32</sup>

## 2. Structures of the Dextrans

### a. Chemical Structure of Dextran Produced by *Leuconostoc mesenteroides*.

Fowler, Buckland, Brauns and Hibbert<sup>34</sup> were the first to investigate systematically the chemical structure of this dextran. On hydrolysis with dilute aqueous sulfuric acid, only D-glucose, in 90% yield, was obtained.

(28) E. E. Snell and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.*, **27**, 1 (1941).

(29) S. Gaines and G. L. Stahly, *J. Bact.*, **46**, 441 (1943).

(30) A. Carruthers and Evelyn A. Cooper, *Biochem. J.*, **30**, 1001 (1936).

(31) W. D. Daker and M. Stacey, *J. Chem. Soc.*, 585 (1939).

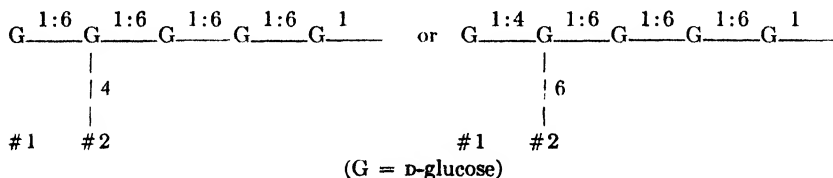
(32) W. Z. Hassid and H. A. Barker, *J. Biol. Chem.*, **134**, 163 (1940).

(33) G. L. Stahly, U. S. Pat. 2,310,263 (1943).

(34) Frances L. Fowler, Irene K. Buckland, F. Brauns and H. Hibbert, *Can. J. Research*, **B15**, 486 (1937).

The presence of three hydroxyl groups per glucose unit was shown by the preparation of a triacetate and a tribenzoate. Six or seven methylations (using dimethyl sulfate and concentrated alkali) of dextran did not raise the methoxyl content above 41% (theoretical maximum 45.6%). Also, Purdie methylations (using methyl iodide and silver oxide) and methylation with thallium ethoxide and methyl iodide were ineffective in raising the methoxyl content of methylated dextran above 43.5%. The maximum theoretical methoxyl content was eventually attained by modified Muskat methylations.<sup>35</sup> Partially methylated dextran suspended in anisole solution was treated with sodium in liquid ammonia, and the sodium salt of methylated dextran thus formed was allowed to react with methyl iodide. The methoxyl content of the partially methylated dextran was raised by three such methylations from 42% to 45.5% and by five such methylations from 30% to 45.4%.

Fully methylated dextran<sup>34</sup> was hydrolyzed by heating with hydrogen chloride (1.5%) in anhydrous methanol in a sealed tube. The mixture of methyl D-glucosides so obtained was fractionated first by differential solubilities in chloroform and water, following the method of Macdonald.<sup>36</sup> In this treatment the methyl dimethyl-D-glucoside dissolved in the water, the methyl tri- and tetra-methyl-D-glucosides in the chloroform. The chloroform-soluble material was fractionated by distillation under reduced pressure. The fractionation results, although not quantitative, indicated a ratio of one part methyl 2,3,4,6-tetramethyl-D-glucoside to three parts methyl 2,3,4-trimethyl-D-glucoside, to one part methyl dimethyl-D-glucoside (identified as methyl 2,3-dimethyl- $\alpha$ -D-glucoside by means of the 6-trityl derivative, the formation of which, however, was not quantitative). These results indicated a cross linkage for every repeating unit of five D-glucose molecules, and 1,6-linkages within each repeating unit. A repeating unit of the following type was suggested as one of the various possibilities for dextran<sup>34</sup>:



D-Glucose residue #1 would therefore appear as 2,3,4,6-tetramethyl-D-glucose on hydrolysis of methylated dextran; D-glucose #2 as 2,3-dimethyl-D-glucose and the other three D-glucose residues as 2,3,4-tri-

(35) I. E. Muskat, *J. Am. Chem. Soc.*, 56, 693, 2449 (1934).

(36) J. Y. Macdonald, *J. Am. Chem. Soc.*, 57, 771 (1935).

methyl-D-glucose. These, however, do not exhaust all the possibilities and it is conceivable that the side chain may consist of two, three or four units with a corresponding shortening of the primary chains.<sup>6</sup>

Hassid and Barker<sup>32</sup> have also reported a similar branched chain structure for a dextran synthesized by a strain of *L. mesenteroides* (synonym *Betacoccus arabinosaceus* Orla-Jensen).

Levi, Hawkins and Hibbert<sup>5</sup> have recently confirmed the structure for *L. mesenteroides* dextran proposed by Fowler, Buckland, Brauns and Hibbert.<sup>34</sup> Completely methylated dextran in an over-all yield of 71% was obtained by three methylations with dimethyl sulfate and sodium hydroxide, followed by six modified Muskat methylations. The methylated dextran was hydrolyzed at 140° with methanolic hydrogen chloride, and the mixture of methylated glucosides, obtained in 95% yield, was quantitatively separated by fractional distillation. 2,3-Dimethyl-D-glucose was identified by means of the phenylhydrazide of the corresponding D-gluconic acid.

*b. Electron Microscope Study of Dextran Produced by Leuconostoc mesenteroides.* Further confirmation of the structure of *L. mesenteroides* dextran (branch chains of maximum length of five D-glucose units) as proposed by Hibbert and coworkers<sup>5,34</sup> is forthcoming from electron microscope studies. Using a magnification of 30,000, Ingelman and Siegbahn<sup>37</sup> obtained evidence that *L. mesenteroides* dextran may exist in thin threads of diameter 30–100 Å. A side chain of five D-glucose units on either side of the main dextran chain would require a total molecular width of ten D-glucose units or 50 Å, and the value of 30–100 Å for the breadth and thickness of the dextran molecule checks within the limits of experimental error. Small nodes were uniformly situated along the fine molecular threads of dextran at intervals of 800 Å, *i. e.*, 160 D-glucose units.

*c. Dextrans of Strains and Variants of Leuconostoc mesenteroides.* The studies of Sugg and Hehre (page 215) on immunological cross reactions of dextrans with pneumococcus antisera have shown that differences exist between dextrans produced by various strains of *L. mesenteroides*. Hibbert and coworkers have also detected differences in physical and chemical properties of *L. mesenteroides* dextrans. Specific viscosity measurements suggested that molecular weights of these dextrans of the same general chemical structures<sup>5,34</sup> might vary by a factor of ten. Such results were inconclusive, however, since specific viscosities varied over a wide range when determined at different concentrations.<sup>3</sup> Differences are also apparent in solubilities, stabilities of dextrans during methylation and in ease of hydrolysis of methylated dextrans.

(37) B. Ingelman and K. Siegbahn, *Nature*, 154, 237 (1944).

It is not clear whether different dextrans are produced by variants of *L. mesenteroides* or by unfavorable cultural conditions. All varieties of *L. mesenteroides*, from those exhibiting smooth to those exhibiting very rough growth, may be isolated from the same source and give identical cultural reactions.<sup>26</sup> The crude dextrans produced by these variants range from gelatinous masses to granular precipitates. These physical variations may be due to differences in molecular aggregation only, as occur with dextrans from different strains of *L. mesenteroides*. However, *Leuconostoc* variants possess different redox potentials, and show different activities with regard to the related capacities of sucrose inversion and dextran formation.<sup>38</sup>

*d. Chemical Structure of Dextran Produced by Leuconostoc dextranicum.* The structure of the dextran produced by the action of *L. dextranicum* was shown by Fairhead, Hunter and Hibbert<sup>39</sup> to be quite different from that of *L. mesenteroides*. The fully methylated dextran was obtained by methylation with dimethyl sulfate and alkali followed by Muskat<sup>5,34</sup> methylations. Hydrolysis of the fully methylated dextran yielded 90% methyl 2,3,4-trimethyl-D-glucosides, and about 10% methyl dimethyl-D-glucosides (not identified), but no methyl 2,3,4,6-tetramethyl-D-glucoside was obtained. Since no evidence for the presence of "end groups" was found, the dextran was considered to be a linear polymer in which the terminal units of one chain were in chemical union with members of adjacent chains to form a network structure.

Peat, Schlüchterer and Stacey<sup>6</sup> later obtained a small amount (0.23%) of methyl 2,3,4,6-tetramethyl-D-glucoside from the hydrolytic products of a methylated *L. dextranicum* dextran, in addition to 90% methyl 2,3,4-trimethyl-D-glucosides, and on the basis of these results, postulated a minimum chain length of 550 units for this dextran. An appreciable amount of methyl dimethyl-D-glucosides was obtained, but these may not indicate side chain linkages since a mixture of dimethyl isomers was present, and the dextran was not fully methylated (only 44.5% methoxyl rather than 45.6%).

### 3. Immunological Properties of Dextrans

*a. Dextrans as Antigens.* Early investigations on the immunological character of the dextrans were carried out mainly from the standpoint of the properties of dextrans as antigens.

Zozaya<sup>40</sup> reported that nitrogen-free *L. mesenteroides* dextran was not antigenic, but could be rendered antigenic by adsorption upon a

(38) V. O. Kagan, *Microbiology (U. S. S. R.)*, **6**, 158 (1937); *C. A.*, **33**, 5881 (1939).

(39) E. C. Fairhead, M. J. Hunter and H. Hibbert, *Can. J. Research*, **B16**, 151 (1938).

(40) J. Zozaya, *J. Exptl. Med.*, **55**, 325 (1932).

colloidal carrier such as collodion (precipitated from acetone solution into water). The production of antibodies was specific, and the antiserum absorbed with the collodion-dextran antigen no longer precipitated this antigen. The antiserum was effective only in relatively low ranges of dilution. Later work indicated that the dextran which had been used was not nitrogen-free.

Zozaya<sup>41</sup> also reported that *L. mesenteroides* dextran reacted immunologically with antisera of pneumococcus, certain of the *Salmonella* group, and some strains of *Streptococcus viridans*(?). Here also these weak cross relations were apparent only in low dilution ranges. Antisera absorbed with the homologous polysaccharides no longer precipitated these homologous polysaccharides, but still precipitated dextran. Antisera absorbed with dextran still precipitated the homologous polysaccharides and dextran, although weakly.

Fitzgerald<sup>42</sup> found that the antibody response of rabbits toward 1.5% aqueous dextran solutions (both types) varied with the nitrogen (*i. e.*, bacterial) content of the dextran. Injections were administered both interperitoneally and intravenously, and no antibody production resulted when the nitrogen content of the dextrans was below 0.2%.

*b. Dextrans as Haptenes.* More recent immunological studies on dextran have been concerned with its haptene character, *i. e.*, the precipitin reaction of dextran with anti-*Leuconostoc* sera prepared with the homologous organisms. Evans, Hawkins and Hibbert<sup>3</sup> obtained precipitin reactions with relatively high dilutions (up to 1/100,000) of dextran, and anti-*Leuconostoc mesenteroides* sera from rabbits. The precipitin reactions obtained with several dextran preparations of different (varying from less than 0.08% to 0.16%) nitrogen content indicated that as the nitrogen content decreased, precipitation occurred at a higher dilution. Since the antigenic activity of the dextran was not dependent on traces of nitrogenous impurity, it was concluded that dextran was a haptene.

Sugg and Hehre<sup>43</sup> also obtained precipitin reactions with dextran or with sterile filtrates of sucrose broth cultures of *L. mesenteroides* (designated for convenience strain A) and not only anti-*Leuconostoc* sera, but also pneumococcus Types II, XII and XX antisera. *Leuconostoc* organisms cultured on D-glucose broth neither stimulated the production of dextran-reactive antibodies in rabbits, nor absorbed dextran-reactive antibodies from sera, as did organisms cultured on sucrose. Absorption with the homologous bacteria (*Leuconostoc*, pneumococcus Types II,

(41) J. Zozaya, *J. Exptl. Med.*, **55**, 353 (1932).

(42) J. G. Fitzgerald, *Trans. Roy. Soc. Can.*, Sect. 5, **27**, 1 (1933).

(43) J. Y. Sugg and E. J. Hehre, *J. Immunol.*, **43**, 119 (1942).

XII or XX) removed the capacities of each antiserum to react with all of the antigens, and absorption with each kind of bacteria removed the capacity of all the antisera to react with antigens of that kind of bacteria.

Dextran resulting from the action of another strain of *L. mesenteroides* (designated for convenience, strain B) was more soluble than strain A dextran and exhibited somewhat different immunological reactions. This strain B dextran reacted only slightly with pneumococcus Type XII antisera and had a narrower zone of antigenic reactivity than the strain A dextran. In addition, the strain A organisms exhibited a greater capacity to absorb antibodies reactive with the B dextran than did the B bacteria to absorb antibodies reactive with the A dextran.

#### 4. Enzymic Synthesis of Dextran

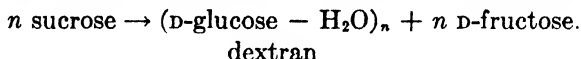
*a. Preparation and Properties of Enzymically Synthesized Dextran.* Hehre and Sugg<sup>8</sup> have described the enzymic synthesis of dextran from sucrose. Since bacteria-free culture filtrates gave only low yields of dextran, the enzyme extract was obtained by precipitation from the chloroform emulsions formed when *L. mesenteroides* cultures were shaken with that solvent. This extract was free of organisms, but not entirely free of dextran; the volume of the extract was about 1% of the volume of the original culture. An active extract was also obtainable by grinding the bacterial cells. Dextran formation by means of the extract occurred over a pH range from 4 to 8, with 5.6 the optimal pH, and over a temperature range from 3° to 37° (optimum 23°). The extract was inactivated after five minutes at 55°.

The most active extracts were obtained<sup>8</sup> from *L. mesenteroides* cultures containing sucrose; extracts prepared from *L. mesenteroides* organisms repeatedly transferred through D-glucose broth were of low potency.<sup>43a</sup> Active extracts in dilutions of 1:2 or 1:4 produced dextran in 5% sucrose solutions after one to two hours at 23°, and at pH 5.6 with acetate buffer; demonstrable amounts of dextran were produced after twenty days with 1/10,000 dilution of the extract. Optimal yields of dextran were less than 5% based on sucrose. Small concentrations of dextran were detected by means of precipitin titrations with pneumococcus antisera Types II or XX (see page 215).

This enzymically synthesized dextran differed from that formed by *L. mesenteroides* organisms only in its lower relative viscosity. Both types of dextran are serologically similar (see page 215). Each reacts with antisera of *Leuconostoc* and of pneumococcus Types II, XX, and XII, and comparable ratios of activity against these antisera were observed.

Similar antisera absorption reactions were also obtained with each dextran.

The over-all equation for dextran synthesis may be represented<sup>8</sup> as:



*b. The Role of Phosphorylation.* Kagan, Lyatker and Tsvasman<sup>44</sup> have reported that *L. mesenteroides* can phosphorolyze sucrose in the presence of inorganic phosphorus to produce D-glucose 1-phosphate. It might be concluded, therefore, that dextran synthesis occurs by phosphorylation, as in the cases of synthetic starch and glycogen. However, Hehre has recently made a comparative study<sup>45</sup> of the actions on sucrose and on D-glucose 1-phosphate of: (a) the enzyme extract<sup>8</sup> prepared from *L. mesenteroides*; and (b) potato phosphorylase. He has shown that D-glucose 1-phosphate is not an intermediate in the formation of dextran from sucrose, and that the enzymic synthesis of dextran requires no phosphorylated sugar.

*c. Symbiosis with Yeast.* An interesting example of symbiotic association of *L. mesenteroides* with *Saccharomyces cerevisiae* has been reported by Stacey.<sup>46</sup> After two successive subcultures from the mixed culture, the maximum yield of dextran was obtained in only forty hours, whereas *L. mesenteroides* in pure culture required ten days for maximum dextran production in this medium.<sup>1</sup> Dextran formation was believed due to exocellular enzyme activity since it occurred with sterile culture filtrates. However, larger yields resulted with unfiltered cultures, probably due to stimulation of the enzyme system by a factor elaborated by living yeast cells.

Dextran formation<sup>47</sup> in media containing sucrose inhibits agglutination of yeast cells by *L. agglutinans*, a genetic mutation of *L. mesenteroides*.

### 5. Industrial Significance of Dextran Produced by *Leuconostoc* Species

*a. Dextran as an Industrial Nuisance.* The presence of dextran in sugar sirups greatly retards filtration and crystallization. As little as 1% dextran more than doubles the viscosity of sucrose solutions, and 6% dextran increases the viscosity thirty-seven times.<sup>48</sup> *Leuconostoc dex-*

(44) B. O. Kagan, S. N. Lyatker and E. M. Tsvasman, *Biokhimiya*, **7**, 93 (1942); *C. A.*, **37**, 4760 (1943).

(45) E. J. Hehre, *Proc. Soc. Exptl. Biol. Med.*, **54**, 240 (1943).

(46) M. Stacey, *Nature*, **149**, 639 (1942).

(47) O. A. Bakushinskaya, *Microbiology (U. S. S. R.)*, **9**, 444 (1940); *C. A.*, **35**, 4057 (1941).

(48) M. A. McCalip and H. H. Hall, *Proc. Intern. Soc. Sugar-Cane Tech.*, **6**, 986 (1938); *C. A.*, **33**, 7141 (1939).



*tranicum* organisms may even cause solidification of 15% cane sugar solutions.<sup>49</sup> Sulfur dioxide has been used to control bacterial growth,<sup>50</sup> and lime to remove dextran from sugar solutions.<sup>51</sup> However, traces of dextran still exist in practically all sucrose preparations, even in "C. P." sucrose reagents.<sup>52</sup>

*b. Industrial Applications of Dextran.* Although dextran is primarily a nuisance in sugar refineries, some industrial applications of dextran have been proposed recently. Dextran has been used<sup>53</sup> as a partial substitute for barley malt; the quality of beer was not affected adversely by 10% or 20% dextran in Pilsener malt. Dextran may be one component of a beverage<sup>54</sup> prepared by lactic acid bacterial fermentation of a milk and cacao base. Consideration has been given also to the use of dextran as a filling for soft-center chocolates.

The preparations of dextran, and certain ethers and esters of dextran have been the subjects of about fifteen recent patents. The dextran (of *L. mesenteroides* or *dextranicum*) may be precipitated first<sup>55</sup> from the culture by the addition of ethanol or acetone, or the culture itself may be treated directly with the appropriate halide and sodium hydroxide<sup>56</sup> in either a single stage<sup>57</sup> or a two-stage procedure.<sup>58</sup> The benzyl ether of dextran, formed by the reaction<sup>57</sup> of dextran with benzyl chloride and sodium hydroxide at 75–185° for three to ten hours, is soluble in acetone and other solvents, and may be used in lacquers.<sup>59</sup> Mixed ether and ester derivatives of dextran, *e. g.*, benzyl and phthalate<sup>60a</sup> or butyl and benzoate,<sup>60b</sup> have been used for forming films, coatings or molded products. A preparation of dextran acetate<sup>64</sup> has also been patented.<sup>60c</sup>

(49) J. A. Alford and C. S. McCleskey, *Proc. Louisiana Acad. Sci.*, **6**, 36 (1942); *C. A.*, **36**, 5046 (1942).

(50) W. Jonáš, *Z. Zuckerind. čechoslovak. Rep.*, **51**, 161, 173 (1927); *C. A.*, **21**, 1564 (1927).

(51) V. Konn, *Listy Cukrovar.*, **48**, 563 (1930); *C. A.*, **24**, 4949 (1930).

(52) J. M. Neill, E. J. Hehre, J. Y. Sugg and E. Jaffe, *J. Exptl. Med.*, **70**, 427 (1939).

(53) M. Hamburg, *Brau- u. Malzind.*, **34**, 15 (1934); *C. A.*, **28**, 7418 (1934).

(54) L. Martens, Swiss Pat. 217,217 (1942).

(55) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,344,179 (1944).

(56) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,203,702-3-4-5 (1940).

(57) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,344,180 (1944).

(58) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,328,036 (1943).

(59) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,236,386 (1941).

(60a) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,229,941 (1941); (b) G. L. Stahly and W. W. Carlson, U. S. Pat. 2,239,980 (1941); (c) W. A. Waldie and J. E. Bersuder, U. S. Pat. 2,344,190 (1944).

### 6. Medical Application of Dextran

Claims have recently been made<sup>61</sup> that the dextran of *L. mesenteroides* may serve as an efficient substitute for blood plasma. Solutions of partially-hydrolyzed dextran in saline gave favorable results when injected intravenously into experimental animals. Preliminary clinical tests were promising.

## IV. THE STRUCTURES OF OTHER BACTERIAL POLYSACCHARIDES

### 1. Miscellaneous Polysaccharides Resembling Dextrans

The structures of a number of other bacterial polysaccharides resembling the dextrans have been investigated.

*Polysaccharides Produced by:*

a. *Betabacterium vermiforme*(?) Ward-Mayer. The polysaccharide (vermiformé dextran) produced from sucrose by this organism was shown<sup>31</sup> to consist of basal chains of twenty-five D-glucose units joined by 1,6-linkages. Osmotic pressure determinations gave a molecular weight of approximately 500 units, while a chain-length of twenty-seven D-glucose units was suggested by the iodine number. Hydrolysis of the methylated polysaccharide yielded methyl 2,3,4-trimethyl-D-glucoside (90%) and methyl 2,3,4,6-tetramethyl-D-glucoside (5%).

b. *Phytophthora tumefaciens*. The polysaccharide<sup>62</sup> produced from sucrose by *Phytophthora tumefaciens* A-6 (crown gall organism) is quite soluble in water and of relatively low molecular weight. Sedimentation velocity and diffusion studies indicated a molecular weight of  $3600 \pm 200$ , i. e., about 22 D-glucose units. Hydrolysis indicated that the polysaccharide is composed entirely of  $\beta$ -D-glucopyranose units.

The optical activity of this polysaccharide in water and cuprammonium solution so closely resembles that of methyl 2-methyl- $\beta$ -D-glucoside that Reeves<sup>63</sup> has suggested that the D-glucopyranose units of the polysaccharide are linked chiefly through the 2-position (see page 231).

c. *Streptococcus bovis*. Certain strains of *Streptococcus bovis* produce dextran-like polysaccharides yielding only D-glucose (e. g., 93%) on hydrolysis.

d. *Streptococcus salivarius*. Some strains of *Streptococcus salivarius* produce mainly levans, together with smaller amounts of a dextran-like polysaccharide.<sup>64</sup> Sterile enzymic extracts which form serologically-

(61) A. Grönwall and B. Ingelman, *Nature*, **155**, 45 (1945).

(62) F. C. McIntire, W. H. Peterson and A. J. Riker, *J. Biol. Chem.*, **143**, 491 (1942).

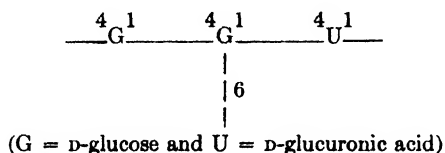
(63) R. E. Reeves, *J. Biol. Chem.*, **154**, 49 (1944).

(64) C. F. Niven, K. L. Smiley and J. M. Sherman, *J. Biol. Chem.*, **140**, 105 (1941).

reactive polysaccharides similar to those produced in the corresponding bacterial cultures, have been prepared<sup>8</sup> from group H streptococci, *Streptococcus salivarius*, and several non-spore-forming bacilli (presumably lactobacilli) isolated from plants.

*e. Rhizobium radicicolum*(?) and *Azotobacter chroococcum*. The polysaccharides produced from sucrose by such nitrogen-fixing organisms as *Rhizobium radicicolum* (clover strain) and *Azotobacter chroococcum* consist of about 67% D-glucose and 23% uronic acid residues, and about 87% D-glucose and 3% uronic acid residues respectively.<sup>65a</sup> Both probably belong to the same class as the specific polysaccharides of pneumococcus Types II and III. Considerably larger yields of polysaccharide are produced by these nitrogen-fixing organisms when cultured on agar<sup>65b</sup> rather than in liquid media. Certain polyhydric alcohols, disaccharides, polysaccharides, organic acids and hexoses form suitable substrates.

In a recent paper<sup>65c</sup> the relationships between *Rhizobium radicicolum* polysaccharides and those of pneumococcus are indicated by cross-precipitin reactions of the former with Types III and VI anti-pneumococcus horse sera. In addition, hydrolysis of the methylated polysaccharide of *Rhizobium radicicolum* yields equal amounts of 2,3,6-trimethyl-D-glucose, 2,3-dimethyl-D-glucose and 2,3-dimethyl-D-glucuronic acid.<sup>65d</sup> The minimum trisaccharide repeating unit consists, in part, of cellobiuronic acid, and may be represented:



*f. Bacillus krzemieniewski*(?). This soil bacillus forms thick, gelatinous capsules on carbohydrate media and yields a polysaccharide which on hydrolysis is stated to produce L-mannose.<sup>65e</sup> This claim is based upon the melting point of the isolated mannose phenylhydrazone and upon the rotation of the hydrolyzed reaction mixture. It requires further confirmation.

(65a) Evelyn A. Cooper, W. D. Daker and M. Stacey, *Biochem. J.*, **32**, 1752 (1938).

(65b) Evelyn A. Cooper and J. F. Preston, *J. Soc. Chem. Ind.*, **56**, 1T (1937).

(65c) H. G. Bray, E. Schlüchterer and M. Stacey, *Biochem. J.*, **38**, 154 (1944).

(65d) E. Schlüchterer and M. Stacey, *J. Chem. Soc.*, 776 (1945).

(65e) A. Kleczkowski and P. Wierzchowski, *Soil Sci.*, **49**, 193 (1940).

## 2. *Pneumococcus Polysaccharides*<sup>65f</sup>

The polysaccharides of pneumococcus (*Diplococcus pneumoniae*) have been the subjects of numerous investigations in the past twenty years.<sup>66</sup> It is possible within the limits of this review to consider only a few of the more important results briefly.

The researches<sup>66</sup> of Heidelberger, Avery, Goebel and coworkers have shown that the capsular carbohydrates of pneumococcus are responsible for immunological type specificity, and that these carbohydrates may function not only as haptenes but also as antigens,<sup>67</sup> if the method of isolation has been sufficiently mild so that no functional groups, *e. g.*, acetyl, are removed. On the contrary, Felton and Prescott<sup>68</sup> concluded that the presence or absence of acetyl groups in Type I pneumococcus polysaccharide prepared by various methods was of no significance in regard to its antigenicity.

The characteristics of the polysaccharides of pneumococcus Types I-XXXII have been studied in some detail.<sup>69</sup> All of these polysaccharides were optically active, twenty-five being dextrorotatory and seven levorotatory. Nine contained uronic acids; seventeen were, partially at least, composed of amino sugars, and three contained slight amounts of amino sugars. Polysaccharides derived from Types I, IV, V, XII and XXV contained up to 5% nitrogen. Removal of nitrogen with nitrous acid resulted in a loss of specificity. Ten polysaccharides contained no nitrogen. The maximum acetyl content was 16%, in the cases of IV and XI. Type I contains O-acetyl only; Type IV contains N-acetyl. A few of the polysaccharides contained phosphorus, the maximum being 6.4% in the cases of Types XXVIII and XXXII. Precipitation titers (with antisera prepared against the corresponding organisms) may be as high as one in eight million. Cross reactions are obtainable with certain types, *e. g.*, III and VIII, but specific inhibition of precipitation (no precipitation of heterologous or homologous antigen in antiserum absorbed with homologous antigen, but precipitation of homologous antigen only in antiserum absorbed with heterologous antigen) also occurs in these cases.

*a. Chemical Structures.* Acid hydrolysis of pneumococcus polysaccharides has revealed something of their chemical structures. The hydrolytic products are D-glucose, D-glycuronic or aldobionic acids and

(65f) Cf. also pages 180, 185 and 189.

(66) B. White, "The Biology of *Pneumococcus*," The Commonwealth Fund, New York (1938).

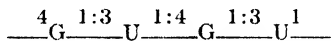
(67) O. T. Avery and W. F. Goebel, *J. Exptl. Med.*, **58**, 731 (1933).

(68) L. D. Felton and B. Prescott, *J. Bact.*, **38**, 579 (1939).

(69) Rachel Brown, *J. Immunol.*, **37**, 445 (1939).

amino sugars. For example,<sup>70</sup> Type I polysaccharide yields 28% D-galacturonic acid and an amino sugar derivative. With methanolic hydrogen chloride, D-galacturonic methyl ester is obtained. Type II polysaccharide yields D-glucose (70%) (reducing sugars after hydrolysis, 95%). Type III polysaccharide yields cellobiuronic acid<sup>71</sup> (reducing sugars after hydrolysis, 85%). Type VIII polysaccharide yields cellobiuronic acid and D-glucose<sup>71</sup> (reducing sugars after hydrolysis, 87%).

Reeves and Goebel<sup>72</sup> have shown that hydrolysis of the reduced methylated capsular polysaccharide of Type III pneumococcus yields 2,3,6-trimethyl-D-glucose and the anomeric forms of methyl 2,4-dimethyl-D-glucoside. The cellobiuronic acid units in the polysaccharide are thus linked through position 3 of the D-glucuronic acid residue, probably by  $\beta$ -D-linkages. That is, the polysaccharide contains alternate 1,3- and 1,4- $\beta$ -D-linkages.



(G represents D-glucose and U represents D-glucuronic acid)

Of great interest is the possible determination of structures of pneumococcus polysaccharides by comparison with a known chemical structure such as dextran. The extent of the cross-precipitation reactions<sup>43</sup> of dextran with various pneumococcus antisera indicates the closeness of the relationships between the structures of dextran and of the various pneumococcus polysaccharides.

### 3. Polysaccharides of Pathogenic Bacteria

Few of these polysaccharides are capsular carbohydrates, and very little is known of their chemical structures. In general, they consist of more than one simple sugar. For example, hydrolysis of the polysaccharide from one strain of cholera organism yields D-glucose and D-galactose. The polysaccharide<sup>73</sup> of one strain of leprosy bacillus is mainly an arabinosan with a small amount of D-galactose. A polysaccharide composed of D-galactose, L-rhamnose, and a *N*-acetyl amino sugar has been isolated<sup>74</sup> from a smooth strain of *Shigella dysenteriae* Shiga. (See page 200).

Varying proportions of D-mannose and of the rarely occurring D-form of arabinose are obtained<sup>75</sup> on hydrolysis of the polysaccharide of tubercle bacillus (*Mycobacterium tuberculosis*) strain H-37, whereas the poly-

(70) M. Heidelberger, W. F. Goebel and O. T. Avery, *J. Exptl. Med.*, **42**, 701 (1925).

(71) R. D. Hotchkiss and W. F. Goebel, *J. Biol. Chem.*, **121**, 195 (1937).

(72) R. E. Reeves and W. F. Goebel, *J. Biol. Chem.*, **139**, 511 (1941).

(73) R. J. Anderson and M. M. Creighton, *J. Biol. Chem.*, **131**, 549 (1939).

(74) W. T. J. Morgan, *Helv. Chim. Acta*, **21**, 469 (1938).

(75) M. Heidelberger and A. E. O. Menzel, *J. Biol. Chem.*, **118**, 79 (1937).

saccharide of strain A-10 in addition consists of D-galactose and inositol.<sup>76</sup> Tuberculin,<sup>77</sup> the concentrated bacteria-free liquid culture in which tubercle bacilli have grown, contains a polysaccharide which yields on hydrolysis D-arabinose, D-mannose, D-galactose and a trace of D-glucosamine. Glycogen preparations of very high particle weight ( $12-13 \times 10^6$ ) have been isolated<sup>78</sup> by sedimentation at high speed of borate buffer or trichloroacetic acid extracts of avian tubercle bacilli; D-glucose was the only product obtained on hydrolysis of this polysaccharide. (See page 201.)

Polysaccharides of *Corynebacterium diphtheriae* yield D-galactose, pentoses and amino sugars on hydrolysis.<sup>79</sup> D-Glucose and D-mannose are major hydrolytic products of the polysaccharide of *Clostridium perfringens*.<sup>80</sup> Complete hydrolysis<sup>81</sup> of the polysaccharide of the anthrax bacillus yielded acetyl-D-glucosamine and D-galactose.

The polysaccharide<sup>82</sup> of *Eberthella typhosa* has a minimum molecular weight of 10,000. It contains small amounts of nitrogen and phosphorus and 6% acetyl. Hydrolysis of this polysaccharide yields reducing sugars consisting of about 50% D-glucose and approximately equal amounts of D-mannose and D-galactose. The polysaccharide of *Salmonella typhimurium*<sup>83</sup> consists of the same three hexoses with a somewhat lesser proportion of D-glucose.

#### 4. Polysaccharides of Molds and Yeasts

Although polysaccharide metabolic products of molds and yeasts are not strictly bacterial polysaccharides, they are considered briefly here because of similarities in chemical structure (see also page 191).

a. *Varianose*. Varianose, produced by the action of the mold *Penicillium varians* G. Smith on D-glucose was found<sup>84</sup> to consist of 6 to 8  $\beta$ -D-galactopyranose units joined by 1,4-linkages with a D-glucopyranose radical at one end, and either L-altrose or D-idose at the reducing end.

b. *Mannocarolose*. This is produced by the action of the mold *Penicillium charlesii* G. Smith on D-glucose.<sup>85</sup> Hydrolysis of the methyl-

(76) R. J. Anderson, R. E. Reeves and F. H. Stodola, *J. Biol. Chem.*, **121**, 649 (1937).

(77) Florence B. Seibert, *Chem. Revs.*, **34**, 107 (1944).

(78) E. Chargaff and D. H. Moore, *J. Biol. Chem.*, **155**, 493 (1944).

(79) E. Chargaff, *Rept. Proc. 3rd Intern. Congr. Microbiol.*, 223 (1939); *C. A.*, **34**, 6321 (1940); E. M. Gubarev, *Biokhimiya*, **7**, 180 (1942); *C. A.*, **38**, 142 (1944).

(80) Muriel H. Svec and Elizabeth McCoy, *J. Bact.*, **48**, 31 (1944).

(81) G. Ivánovics, *Z. Immunitäts.*, **98**, 420 (1940); *C. A.*, **38**, 3344 (1944).

(82) G. G. Freeman and T. H. Anderson, *Biochem. J.*, **35**, 564 (1941); G. G. Freeman, *ibid.*, **36**, 340 (1942).

(83) G. G. Freeman, *Biochem. J.*, **37**, 601 (1943).

(84) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, **29**, 2668 (1935).

(85) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, **29**, 612 (1935).

ated derivative and conversion to the mannosides was reported to give methyl 2,3,4-trimethyl-D-mannoside (75%), methyl 2,3,4,6-tetramethyl-D-mannoside (13.4%) and methyl dimethyl-D-mannoside (10%). The last substance was due presumably to incomplete methylation. The authors concluded from these results that mannocarolose consisted of 8 to 9 D-mannopyranose units linked through the 1,6-positions. However, 2,3,4-trimethyl-D-mannose has since<sup>86</sup> been synthesized and is quite different from the trimethylmannose isolated from the hydrolytic products of methylated mannocarolose. The structure of the latter is, therefore, still unknown.

c. *Galactocarolose*. This polysaccharide is also produced by *Penicillium charlesii* G. Smith from D-glucose and was investigated by the same authors.<sup>87</sup> Hydrolysis of methylated galactocarolose produced methyl 2,3,5,6-tetramethyl-D-galactofuranoside (12.4%), and methyl 2,3,6-trimethyl-D-galactoside (80%). These results point to a chain of 9 to 10 D-galactose units, joined through the 1,5-positions.

d. *Luteose*. The polyglucose luteose<sup>88</sup> occurring as the malonyl ester (luteic acid) is a metabolic product of *Penicillium luteum* Zukal cultured on any of a variety of common sugars. Hydrolysis of luteose yields only D-glucose, and hydrolysis of trimethyl luteose with fuming hydrochloric acid yields mainly 2,3,4-trimethyl-D-glucose with about 10% dimethyl-D-glucoses (probably due to incomplete methylation) but no tetramethyl-D-glucose. Luteose thus appears to consist of a chain of  $\beta$ -D-glucose units joined by 1,6-linkages (cf. *L. dextranicum* dextran, page 214). Another polysaccharide, galuteose, formed under the same conditions as luteose, consists mainly of D-galactose and D-mannose.

e. *Yeast Mannan*. The structure of yeast mannan was investigated by Haworth and coworkers.<sup>89</sup> Hydrolysis of methylated mannan yielded approximately equimolecular proportions of tetramethyl-D-mannose, 3,4-dimethyl-D-mannose and trimethyl-D-mannose. The latter consisted of approximately equal amounts of 3,4,6- and 2,4,6-trimethyl-D-mannoses, with less than 10% of 2,3,4-trimethyl-D-mannose. Thus yeast mannan consists of D-mannose units joined by 1,2- 1,3- and 1,6-linkages (probably

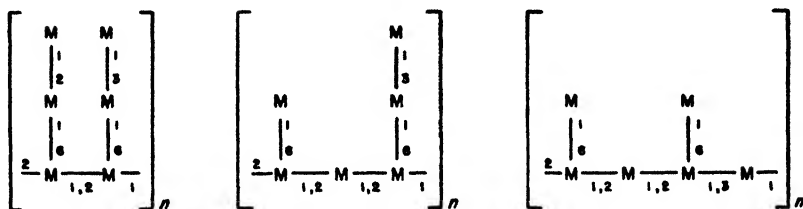
(86) W. N. Haworth, E. L. Hirst, F. A. Isherwood and J. K. N. Jones, *J. Chem. Soc.*, 1878 (1939).

(87) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, 31, 640 (1937).

(88) C. G. Anderson, W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, 33, 272 (1939).

(89) W. N. Haworth, E. L. Hirst and F. A. Isherwood, *J. Chem. Soc.*, 784 (1937); W. N. Haworth, R. L. Heath and S. Peat, *ibid.*, 833 (1941).

$\alpha$ -D). The branched chain structure is one of the following types:



(where M = D-mannose and the numerals indicate the points of mutual linkage)

*f. Yeast Insoluble Polysaccharide.* The structure of an insoluble polysaccharide from the yeast *Saccharomyces cerevisiae* was investigated by Zechmeister and Toth,<sup>90a</sup> and also by Hassid, Joslyn and McCready.<sup>90b</sup> The isolation<sup>90b</sup> of 2,4,6-trimethyl-D-glucose as the sole product of the hydrolysis of the methylated polysaccharide indicated a chain of glucopyranose units joined by 1,3-glucosidic linkages.

Per-iodic acid oxidation (see page 232) has confirmed the presence of 1,3-glucosidic linkages in this yeast glucan, and has indicated one end-group for each twenty-eight D-glucose units.<sup>91</sup> Enzymic hydrolysis showed that the glucosidic linkages were of the  $\beta$ -D-configuration.

*g. Levan.* Levan has been produced by enzyme preparations from the mold *Aspergillus sydowi*.<sup>92</sup>

*h. Polysaccharide of Coccidioides immitis.* An immunologically active (skin reactions, precipitative reactions) polysaccharide formed by the fungus *Coccidioides immitis* has been investigated.<sup>93</sup> Hydrolysis of this polysaccharide produced D-glucose, D-galacturonic acid and an unidentified nitrogen-containing sugar in the approximate ratio 6 : 1 : 3.

## V. LEVANS

### 1. Early Studies

The occurrence of fructosans in plants is widespread (see page 253).<sup>94</sup> The formation of a bacterial fructosan, levan, by a number of different organisms has also been investigated.

(90a) L. Zechmeister and G. Toth, *Biochem. Z.*, **284**, 133 (1936).

(90b) W. Z. Hassid, M. A. Joslyn and R. M. McCready, *J. Am. Chem. Soc.*, **63**, 295 (1941).

(91) V. C. Barry and T. Dillon, *Proc. Roy. Irish Acad.*, **49B**, 177 (1943).

(92) N. Kopeloff, Lillian Kopeloff and C. J. Welcome, *J. Biol. Chem.*, **43**, 171 (1920).

(93) W. Z. Hassid, E. E. Baker and R. M. McCready, *J. Biol. Chem.*, **149**, 303 (1943).

(94a) H. Hibbert, R. S. Tipson and F. Brauns, *Can. J. Research*, **4**, 221 (1931);

(b) W. Z. Hassid, *J. Am. Chem. Soc.*, **61**, 1223 (1939).



Early reports on levan are obscured by incomplete descriptions of impure products.<sup>25, 95</sup> Greig-Smith found that *Bacillus levaniformans*(?) produced levan from sucrose<sup>96a</sup> in suitable nutrient solutions, but not from D-glucose, D-fructose, lactose or maltose.<sup>96b</sup> He therefore assumed that levan could only be formed from the "nascent" D-fructose and D-glucose resulting from the inversion of sucrose. Hydrolysis of levan yielded D-fructose only, and analysis of levan agreed with the empirical formula  $(C_6H_{10}O_5)_n$ ; it was noted that levan was closely related to inulin but was not identical with it.

Owen<sup>97</sup> conducted experiments on sucrose inoculated with yeast and with organisms of the potato group, *e. g.*, *B. mesentericus*, *B. vulgatus*, and found that levan formation was very markedly reduced by excessive inversion in the presence of the yeast (or of yeast invertase) or by the addition of excess invert sugar. Apparently the "nascent" D-fructose liberated by yeast is not in a suitable condition for polymerization to levan. On the other hand (page 217), symbiotic association of *L. mesenteroides* with *Saccharomyces cerevisiae* produces much larger yields of dextran.<sup>46</sup>

Harrison, Tarr and Hibbert<sup>95</sup> investigated the production of levan from sucrose by the action of *Bacillus subtilis* Cohn and *B. mesentericus* Trevisan. Nutrient solutions containing 10% carbohydrate, 0.1% peptone, 0.2% disodium hydrogen phosphate and 0.5% potassium chloride were incubated at 37° for six days. Levan formation occurred only with sucrose and raffinose, and not with melezitose, lactose, maltose, D-xylose, D-glucose or D-fructose. It was therefore suggested that only those carbohydrates with a terminal D-fructofuranose residue were satisfactory substrates for levan formation.

However, some carbohydrates with a terminal D-fructofuranose residue are not suitable substrates for levan formation, *e. g.*, methyl  $\beta$ -D-fructofuranoside and inulin,<sup>64</sup> even in the presence of enzymes<sup>98</sup> which hydrolyze these substrates.

Hestrin, Avineri-Shapiro and Aschner<sup>9</sup> report that levan formation by means of *B. subtilis* is greater if cultural products other than levan are continually removed by dilution or dialysis. The organism was inoculated into a phosphate-buffered solution contained in a cellophane bag suspended in a large volume of sucrose peptone solution.

(95) F. C. Harrison, H. L. A. Tarr and H. Hibbert, *Can. J. Research*, **3**, 449 (1930).

(96a) R. Greig-Smith, *Proc. Linnean Soc. N. S. Wales*, **26**, 589 (1901).

(96b) R. Greig-Smith and T. Steel, *J. Soc. Chem. Ind.*, **21**, 1381 (1902).

(97) W. L. Owen, *J. Bact.*, **8**, 421 (1923).

(98) S. Hestrin and S. Avineri-Shapiro, *Biochem. J.*, **38**, 2 (1944); S. Hestrin, *Nature*, **154**, 581 (1944); S. Avineri-Shapiro and S. Hestrin, *Biochem. J.*, **39**, 167 (1945).

## 2. Chemical Structure

The structure of the levan synthesized by the action of *B. subtilis* on sucrose was determined by Hibbert and Brauns.<sup>99</sup> Levan, in a yield of 60–65% calculated on the D-fructose part of the sucrose, was obtained by precipitation of the concentrated culture into methanol, and purified by reprecipitation and electrodialysis. Hydrolysis of purified levan with 0.5% aqueous oxalic acid for one hour at 100° gave a 99% yield of crystalline D-fructose. Triacetyllevan was prepared by treatment with acetic anhydride in pyridine, and deacetylation with alcoholic alkali yielded material identical with the original levan.<sup>94a</sup>

Two methylations of levan by means of dimethyl sulfate and potassium hydroxide (not sodium hydroxide) followed by one methylation with methyl iodide and silver oxide yielded trimethyllevan in 88% yield. This was purified by solvent fractionation; over-all yield 75%, m. p. 145–146°, OCH<sub>3</sub> 45.9%. Trimethyllevan, hydrolyzed by heating at 95° for twenty-four hours with a dilute solution of sulfuric acid gave a 98.5% yield of a crystalline trimethyl-D-fructose. The trimethyl-D-fructose was proved<sup>94a</sup> to be 1,3,4-trimethyl-D-fructofuranose since oxidation with nitric acid gave a 97% yield of a dimethyl-2-keto-D-glucosaccharic acid which was identified as the crystalline diamide (95% yield).

Levan is thus a polymerized D-fructofuranose with linkages at positions 2 and 6 of the D-fructose units, thus differing from inulin which is a polymerized fructofuranose with linkages at positions 1 and 2.

The levan synthesized by *B. subtilis* from raffinose was shown by Mitchell and Hibbert<sup>100</sup> to be identical in structure with that obtained from sucrose.

Challinor, Haworth and Hirst<sup>101</sup> determined the chemical structure of the levan produced by the action of *B. mesentericus* on sucrose. Methylated levan appeared homogeneous when fractionally precipitated from mixed solvents. Fractional distillation of the hydrolytic products of methylated levan yielded tetramethyl-D-fructofuranose in an amount corresponding to a levan chain length of from ten to twelve fructofuranose units, joined as previously<sup>94a</sup> shown through the 2- and 6-positions.

Other levans produced by widely different organisms all have similar structures, *e. g.*, the levans<sup>102a</sup> produced from sucrose by *B. megatherium*, *Phytomonas pruni* and *P. prunicola*, and those<sup>102b</sup> produced from sucrose

(99) H. Hibbert and F. Brauns, *Can. J. Research*, **4**, 596 (1931).

(100) W. Mitchell and H. Hibbert, *Can. J. Research*, **7**, 345 (1932).

(101) S. W. Challinor, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 676 (1934).

(102a) R. R. Lyne, S. Peat and M. Stacey, *J. Chem. Soc.*, 237 (1940).

(102b) S. Veibel, *Biochem. J.*, **32**, 1949 (1938).

or raffinose by Gram-negative milk and soil *Actinomycetes*. Certain differences in the properties of the levans produced by different organisms are probably due to varying degrees of aggregation of the repeating unit. Variation in levan particle size is also indicated by sedimentation, ultramicroscope and electron microscope studies.<sup>37</sup> It is not clear whether the presence<sup>101</sup> or absence<sup>94a</sup> of terminal groups indicates a difference in levans from different sources, or merely a difference in techniques and interpretations of data. Levan formation by means of various other organisms<sup>103</sup> has been reported, *e. g.*, *Streptococcus salivarius* and *S. bovis*.<sup>64</sup> Cooper<sup>103a</sup> has suggested that the levan formation test may be valuable, on account of its simplicity, in determinative bacteriology for the identification of species.

### 3. "Starchless Potato"

Cultures of *B. subtilis* were introduced into the stems of young potato plants by Suit and Hibbert<sup>104</sup> in an attempt to bring about replacement of starch by another polysaccharide. Sections of some of the resulting potatoes gave little or no color with iodine, and were provisionally designated "starchless potatoes." However, based on analogy with recent developments in starch chemistry, it seems probable that the "starchless potato" was free from amylose, and contained only amylopectin.

### 4. Enzymic Production

*a. Bacillus mesentericus.* Evidence of the possible synthesis of levan by enzymes of *B. mesentericus* was obtained at an early date.<sup>105</sup> Enzyme suspensions prepared by precipitation into alcohol of the triturated organisms yielded levan, but even in the presence of toluene, these enzyme preparations were not free from bacteria.<sup>95</sup> Sterile filtrates of *B. mesentericus* cultures also yielded levan.<sup>9, 95</sup> Hibbert, Tipson and Brauns<sup>94a</sup> showed that the structure of enzymically synthesized (*B. mesentericus*) levan was identical with the levan produced by *B. subtilis*.

*b. Levansucrase.* Hestrin, Avineri-Shapiro and Aschner<sup>9</sup> have recently reported levan formation by means of bacteria-free enzyme preparations (levansucrase) from *B. subtilis* Marburg, *B. polymyxa* Migula and *Aerobacter levanicum*(?). *B. subtilis* levansucrase could be separated from living cells by selective diffusion through nutrient agar

(103a) Evelyn A. Cooper, *J. Soc. Chem. Ind.*, **58**, 229 (1939); (b) Evelyn A. Cooper and J. F. Preston, *Biochem. J.*, **29**, 2267 (1935).

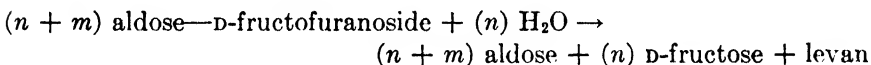
(104) R. F. Suit and H. Hibbert, *Science*, **79**, 78 (1934).

(105) M. W. Beijerinck, *J. Soc. Chem. Ind.*, **29**, 710 (1910).

(sucrose, peptone, phosphate); little or no enzyme was produced in liquid media or in the absence of sucrose in solid media. Sterile levansucrase preparations were obtained from *A. levanicum* by immersion of the organisms in ethanol and ether followed by rapid desiccation *in vacuo*, or by autolysis of the organisms in water in the presence of small amounts of thymol and chloroform. Enzyme preparations retained 90% of their activity after six days at 37°, but were completely inactivated in less than five minutes at 100°. *A. levanicum* produced levansucrase not only from sucrose but also in the presence of invert sugar; *i. e.*, levan is not essential for levansucrase formation. Yields of levan were as high as 60% based on the D-fructose part of the sucrose.

Hestrin and Avineri-Shapiro<sup>98</sup> have recently suggested a mechanism for levan production from sucrose and raffinose by levansucrase. The enzyme was used in the form of the autolyzate from *A. levanicum* (rendered sterile with chloroform and thymol<sup>9</sup>). This autolyzate was incubated for twenty-four hours at 37° with four volumes of 3% sucrose solution and one volume of phosphate buffer (pH 5.0).

It was found<sup>98</sup> that the amount of sucrose consumed could be accounted for entirely as levan and reducing sugars. Fructose formed part of the reducing sugars produced, and there was no appreciable interconversion of aldose and ketose. When raffinose was used as substrate, the products were levan, melibiose and D-fructose (but not sucrose and D-galactose). The following equation expresses fully the net result of the action of the autolyzate:



where *m* and *n* are moles of substrate, aldose is D-glucose (from sucrose substrate) or melibiose (from raffinose), and levan represents a polymer from *m* D-fructofuranose residues of substrate. The ratio *m/n* varied with the conditions of the experiment and the enzyme preparation, but usually had a value of about 1.05 to 1.1, *i. e.*, about one mole of substrate was hydrolyzed for each mole converted to levan and aldose.

The specificity of levansucrase<sup>98</sup> is dependent not only on the D-fructoside but also on the aldose residue of the substrate. Neither inulin nor methyl D-fructofuranoside was hydrolyzed by levansucrase, and even when these two substrates were hydrolyzed by inulase (prepared from inulin-fermenting *Torula* yeast) or by yeast invertase respectively, no levan formation occurred with levansucrase. However, neither methyl D-fructofuranoside nor inulin inhibited levan formation from sucrose by levansucrase. No levan was formed from potassium D-glucose

1-phosphate, sodium D-fructose 1,6-diphosphate, or calcium D-fructose 6-phosphate (except in the presence of sucrose).

Levan formation from sucrose by levansucrase<sup>98</sup> was inhibited (25–75%) by various sugars in concentrations about one-third less than that of the sucrose in the medium.<sup>105b</sup> Inhibition was caused by both sugars and glycosides, *e. g.*, D-glucose, methyl  $\alpha$ -D-glucoside and others which have a configuration about carbon atom 2 similar to that of D-glucose such as D-galactose, maltose, lactose, etc. However, no appreciable inhibition occurred for products like D-mannose, D-fructose, D-mannitol, for which configuration or structure at carbon atom 2 differs from that of D-glucose. The inhibition of levan production from sucrose by free D-glucose corresponded in general form to inhibition by competition for the enzyme; complete inhibition of levan production from 1% sucrose was approached only when the D-glucose concentration was about 16%.

The exact nature of levansucrase activity<sup>98</sup> is not clear. It differs in certain respects from invertase, polymerase, fructosaccharase, and phosphorylase. Possibly the aldose part of the substrate molecule is replaced by an enzyme-linked group, and partial decomposition of this levan precursor to aldose and ketose may furnish the energy necessary for levan synthesis.

Indirect evidence of the reversal of enzymic synthesis of levan has recently been obtained.<sup>105a</sup> Enzyme preparations from *B. subtilis* were shown to contain an enzyme capable of hydrolyzing levan (in addition to the levan-synthesizing enzyme) in a system composed of D-glucose, levan, yeast invertase (to hydrolyze sucrose formed) and the *B. subtilis* enzyme preparation.

*c. Streptococcus salivarius.* Levan has also been synthesized from sucrose and from raffinose by means of *Streptococcus salivarius* enzyme preparations.<sup>105b, 8</sup>

### 5. Immunological Properties

Hehre, Genghof and Neill<sup>105c</sup> have shown that levans isolated from sucrose cultures of *Streptococcus salivarius* (and of a spore-forming bacterium) exhibit precipitation and complement fixation when tested against antisera produced by immunization with bacteria obtained from sucrose cultures of either species.

Similar serological properties are possessed by the levans synthesized by enzymic preparations from these organisms.<sup>105b</sup>

(105a) M. Doudoroff and R. O'Neal, *J. Biol. Chem.*, **159**, 585 (1945).

(105b) E. J. Hehre, *Proc. Soc. Exptl. Biol. Med.*, **58**, 219 (1945).

(105c) E. J. Hehre, Dorothy S. Genghof and J. M. Neill, *J. Immunol.*, **51**, 5 (1945).

## VI. MISCELLANEOUS METHODS FOR DETERMINATION OF POLYSACCHARIDE STRUCTURES

A number of recent papers have described methods which will undoubtedly become increasingly important as tools for the structural determination of bacterial and other polysaccharides.

### 1. *Optical Rotation in Cuprammonium Solution*

Reeves<sup>63</sup> has made the interesting observation that the shift in optical rotation of D-glucopyranose polysaccharides upon being dissolved in cuprammonium hydroxide solution may be used to classify D-glucose polysaccharides, and may in certain instances furnish information regarding the structure of the polysaccharide. The four possible methyl monomethyl- $\beta$ -D-glucopyranosides showed widely different optical behavior when dissolved in cuprammonium hydroxide solution. When the optical rotation in water was compared with that in cuprammonium hydroxide solution, it was found that the latter caused: (1) little change in the rotation of methyl 3-methyl- $\beta$ -D-glucoside or of a 3-linked polysaccharide, *e. g.*, laminarin; (2) a moderately large dextro shift in the rotation of methyl 6-methyl- $\beta$ -D-glucoside; (3) a large dextro shift in the rotation of methyl 2-methyl- $\beta$ -D-glucoside; and (4) a large levo shift in the rotation of methyl 4-methyl- $\beta$ -D-glucoside and of 4-linked polysaccharides, *e. g.*, cellulose, glycogen and soluble starch.

### 2. *Hydrolysis with Hydrogen Bromide in Glacial Acetic Acid*

A solution of hydrogen bromide in glacial acetic acid and acetyl bromide has been used by Jeanes and Hilbert<sup>106</sup> to distinguish qualitatively between 1,4- and 1,6-glycosidically linked disaccharides and polysaccharides. Polarimetric observation indicated that the octaacetates of maltose, lactose and cellobiose were almost completely (83–99%) converted to the acetylated glycosyl halides, whereas gentiobiose and melibiose octaacetates were not hydrolyzed under these conditions. The acetates of various fractions of starch were converted incompletely to acetobromo-D-glucose, in proportion to the degree of branching believed present. The acetate of *L. mesenteroides* dextran, which is composed predominantly<sup>6, 34</sup> of 1,6- $\alpha$ -D-glucosidic linkages yielded no acetobromo-D-glucose on treatment with the hydrogen bromide reagent.

(106) Allene Jeanes and G. E. Hilbert, papers presented before the Division of Sugar Chemistry and Technology, American Chemical Society, New York, September, 1944.

### 3. *Per-iodic Acid Oxidation*

The per-iodic acid oxidation technique may also be used to distinguish between various possible polysaccharide linkages.<sup>107</sup> If the linkage between the units of the polysaccharide is 1,4-, the glycol in the 2,3-positions is cleaved by per-iodic acid; if the linkage is 1,3-, no glycol is available for reaction except in the terminal non-aldehydic group. In the latter case, an indication of chain length is thus obtained.

### 4. *Chromatographic Adsorption*

Chromatographic adsorption appears to offer a simpler method of separation of the hydrolytic products of methylated polysaccharides than fractional distillation. Small amounts of methyl tetramethyl-D-glucosides are quantitatively separable from methyl trimethyl-D-glucosides by adsorption on alumina.<sup>108</sup> A quantitative separation into pure components of a mixture of tetramethyl-D-glucose, 2,3,6-trimethyl-D-glucose (in large excess) and dimethyl-D-glucoses is obtained by partition between organic solvents, such as chloroform, and water held in a column of silica gel,<sup>109a</sup> or by adsorption of benzene-acetone solutions of similar mixtures of methylated glucoses on alumina.<sup>109b</sup>

The hydrolysis products of methylated disaccharides may be separated by *p*-phenylazobenzoylation followed by chromatographic adsorption, or the methanolysis products of the methylated disaccharides may be separated due to differential water solubilities of the *p*-phenylazobenzoates.<sup>110</sup>

Various fully-acetylated monosaccharides and disaccharides or sugar alcohols are readily separable by chromatographic adsorption on Magnesol (a hydrated magnesium acid silicate)<sup>111</sup> and the unacetylated substances may be separated on clay columns.<sup>112</sup>

(107) V. C. Barry, T. Dillon and Winifred McGettrick, *J. Chem. Soc.*, 183 (1942); V. C. Barry, *ibid.*, 578 (1942).

(108) J. K. N. Jones, *J. Chem. Soc.*, 333 (1944).

(109a) D. J. Bell, *J. Chem. Soc.*, 473 (1944).

(109b) Ethelda J. Norberg, I. Auerbach and R. M. Hixon, *J. Am. Chem. Soc.*, 67, 342 (1945).

(110) G. H. Coleman, D. E. Rees, R. L. Sundberg and C. M. McCloskey, *J. Am. Chem. Soc.*, 67, 381 (1945).

(111) W. H. McNeely, W. W. Binkley and M. L. Wolfrom, *J. Am. Chem. Soc.*, 67, 527 (1945).

(112) B. W. Lew, M. L. Wolfrom and R. M. Goepp, Jr., *J. Am. Chem. Soc.*, 67, 1865 (1945); *ibid.*, 68, 1449 (1946).

## VII. CONCLUSION

The marked advances made in the study of bacterial polysaccharides serve only to emphasize the vast amount of work still to be carried out in this field. The recent remarkable achievements in enzymic chemical synthesis (starch, crystalline sucrose, etc.) indicate the growing necessity for greater cooperation<sup>113</sup> between enzymologist and chemist in the solution of many biological phenomena.

(113) M. L. Huggins, *Am. Scientist*, **31**, 338 (1943).





# THE CHEMISTRY OF PECTIC MATERIALS

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### I. INTRODUCTION: GENERAL CHARACTERISTICS OF PECTIN

The elucidation of the general chemical structure of the polysaccharides starch and cellulose was dependent upon the results of investigations into the detailed structure of the simple sugars. In a similar way, knowledge of the fine structure of those groups of polysaccharides, which are more complex than starch and cellulose only in the sense that they are built up of more than one type of sugar residue, is dependent upon the elaboration of experimental technics developed for the investigation of starch and cellulose. It requires also the elaboration of methods<sup>1a</sup> for the separation of closely related sugar derivatives, and if there had been no other reasons than these, it would not have been surprising that attempts to investigate the detailed structures of members of the groups of hemicelluloses and pectic materials were of comparatively recent growth. There are, however, further complications which arise from the colloidal character of these polysaccharides and close association in nature with other carbohydrate materials, which result in isolation and purification problems of unusual difficulty. Some of these so-called hemicelluloses are of relatively simple structure in that they contain residues of one or two sugars or uronic acids only, whilst others—for example, the plant gums—contain a wide variety of sugar residues linked together in the most diverse manner.<sup>1,2</sup> Pectin may

(1) A. G. Norman, "The Biochemistry of Cellulose, The Polyuronides, Lignins, etc." Oxford University Press, London (1937).

(1a) Cf. E. Anderson and Lila Sands, *Advances in Carbohydrate Chem.*, **1**, 329 (1945).

(2) E. L. Hirst, *J. Chem. Soc.*, 70 (1942).

formally be considered a member of the hemicellulose group. It was one of the first to be examined, attention being attracted to it because of its relative ease of isolation, its commercial value and its importance in plant physiology. Indeed, some of the earliest chemical investigations on pectin were instituted because of its botanical interest.<sup>3</sup> The part it plays in the ripening of fruit<sup>4</sup> and in the retting of flax,<sup>5</sup> to mention only two examples, have continued to attract attention, with the result that a large and important literature has now been established. In this brief review, however, it is the authors' intention to concentrate almost wholly on problems concerning the detailed chemical structure of the components of pectic materials.

Pectin occurs in practically all plant materials, especially in fruit and young tissues<sup>6</sup> and it also occurs in wood,<sup>7</sup> but it must be understood that the general term "pectin" is used to describe products which vary greatly in composition and possibly also in chemical constitution, so that it is difficult to frame a rigid definition. Very different pectic products may indeed be isolated from the same plant at different stages of growth.<sup>8,1</sup> The difficult problem then arises as to whether pectins are physical mixtures of polysaccharides or whether they consist mainly of highly complex polysaccharides, the constitution of which varies from source to source. The problem is not yet completely solved, but for reasons which will be discussed below, the former possibility would appear to be more probable. All the pectins so far isolated give, on hydrolysis, in various proportions L-arabinose, D-galactose, D-galacturonic acid and methyl alcohol.<sup>1</sup> Pectins from the younger plants give, on hydrolysis, a somewhat greater proportion of D-galactose and L-arabinose, whilst pectic materials from older and riper sources yield a higher proportion of D-galacturonic acid. In the case of fruits, however, the quantity of pectic material itself tends to disappear owing to enzymic hydrolysis as the fruit becomes over-ripe. In addition, it is considered by some chemists that D-xylose, L-fucose, acetic acid and acetone are combined into the general structure of certain

(3) E. Frémy, *J. pharm. chim.*, **3**, 12 (1847).

(4) C. L. Hinton, "Fruit Pectins. Their Chemical Behaviour and Jellying Properties." Department of Scientific and Industrial Research (1939).

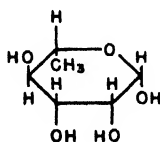
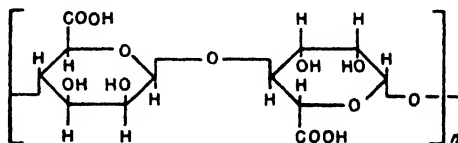
(5) F. Ehrlich and R. Haensel, *Cellulosechem.*, **16**, 97 (1935); F. Ehrlich and F. Schubert, *Biochem. Z.*, **169**, 13 (1926); M. Lüdtke and H. Felser, *Ann.*, **549**, 1 (1941).

(6) Ref. 4, p. 19.

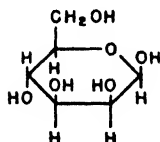
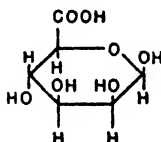
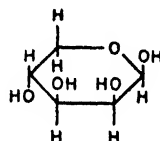
(7) E. Anderson, *J. Biol. Chem.*, **112**, 531 (1936); A. G. Norman, *Biochem. J.*, **25**, 200 (1931).

(8) T. N. Morris, Report of the Food Investigation Board for the Year 1934, p. 200. H. M. Stationery Office, London (1935).

pectins, but the evidence in this connection is still somewhat conflicting.<sup>9</sup> It is interesting to note that pectin so far has not been isolated from marine sources, but instead there is a corresponding polysaccharide, namely alginic acid, which is built up of condensed D-mannuronic acid units.<sup>2</sup> Frequently associated with alginic acid there are polysaccharides derived from L-rhamnose residues<sup>10</sup> and from L-fucose residues, respectively.<sup>11</sup> Up to the present no polysaccharides built up of condensed D-mannose or D-lyxose residues corresponding respectively to the galactan and araban components of pectin have been detected in association with alginic acid. However, sulfuric acid esters of galactans have been encountered.<sup>12</sup>

 $\beta$ -L-Rhamnose

Alginic acid

 $\beta$ -D-Mannose $\beta$ -D-Mannuronic acid $\beta$ -D-Lyxose

Pectins occur naturally in both soluble and insoluble forms. Soluble pectin occurs in plant juices and is particularly abundant in those juices which form jellies, such as black-currant and gooseberry. Insoluble pectins tend to occur in the green parts of plants, in fruit and in root crops.<sup>13</sup> This insolubility is apparently due either to the presence of the pectin as its insoluble calcium or magnesium salt or because it is combined (esterified?) with cellulose or some other insoluble polysac-

(9) R. Bauer, *Chem. Zentr.*, **72**, II, 196 (1901); T. von Fellenberg, *Biochem. Z.*, **85**, 118 (1918); F. Tutin, *Biochem. J.*, **15**, 494 (1921); F. Ehrlich and A. Kosmahly, *Biochem. Z.*, **212**, 162 (1929); K. Smolenski, *Roczniki Chem.*, **4**, 72 (1924).

(10) M. M. T. Plant and E. D. Johnson, *Nature*, **147**, 390 (1941).

(11) G. Lunde, *Papir-J.*, **28**, 147 (1940).

(12) W. Z. Hassid, *J. Am. Chem. Soc.*, **55**, 4163 (1933); *ibid.*, **57**, 2046 (1935); A. Forbes and E. G. V. Percival, *J. Chem. Soc.*, 1844 (1939); V. C. Barry and T. Dillon, *Proc. Roy. Irish Acad.*, **50**, 349 (1945).

(13) T. K. Gaponenkov, *J. Applied Chem. (U. S. S. R.)*, **7**, 1037 (1934).

charide of high molecular weight.<sup>14</sup> The soluble form of pectin consists mainly of the methyl ester of pectic acid, together with galactan and araban. The function of the ester group is uncertain but the carboxyl group may be esterified enzymically in the plant (by pectase) to prevent the development of undue acidity and to render the polysaccharide soluble. On the other hand, the presence of methoxyl groups does not appear to be essential for the formation of pectin gels. The presence of a weak acid and of a polyhydroxy compound containing primary hydroxyl groups does, however, seem to be necessary. Whether gel formation is occasioned by cross linking of the pectic acid molecules through polyhydroxy compounds or whether it is due to the aggregation of de-esterified pectic acid molecules is unknown. The formation of gels has been used to determine the approximate molecular size of pectic acid.<sup>15</sup>

The role of pectin in plant physiology is still far from clear. It is considered by some to be a cementing material but it is evident that the various types of pectic materials are able to perform in plants exceedingly varied biological functions.

## II. EARLY WORK ON THE STRUCTURE OF PECTIN

In spite of the very considerable attention which has been paid to the chemistry of pectic materials, the unpromising physical and chemical properties of the substances concerned have made progress slow. Only very recently, for example, the view put forward by F. Ehrlich that pectin was a polysaccharide of low molecular weight was still generally held.<sup>16,1</sup> However, from the investigations of Smolenski and Wlostouska,<sup>17</sup> Baur and Link<sup>18</sup> and Schneider and Bock<sup>19</sup> it became apparent that pectins were to be regarded as mixtures of polysaccharides of high molecular weight built on the same structural principles as cellulose and other polysaccharides. For example, the structure for pectic acid postulated by Schneider is a long chain of D-galacturonic acid residues in the pyranose form united through positions 1 and 4 (see Fig. 4).

F. Ehrlich was one of the first workers to attempt a detailed chemical investigation of pectin.<sup>20</sup> He subdivided pectic materials into groups;

(14) F. Ehrlich, *Cellulosechem.*, 11, 140 (1930).

(15) H. W. Buston and H. R. Nanji, *Biochem. J.*, 26, 2094 (1932).

(16) D. R. Nanji, F. J. Paton and A. R. Ling, *J. Soc. Chem. Ind.*, 44, 253 (1925).

(17) K. Smolenski and V. Wlostouska, *Roczniki Chem.*, 6, 743 (1926); *ibid.*, 7, 591 (1927); *Chem. Zentr.*, 98, I, 2980 (1927); 99, II, 439 (1928).

(18) L. Baur and K. P. Link, *J. Biol. Chem.*, 109, 293 (1935).

(19) G. G. Schneider and H. Bock, *Ber.*, 70, 1617 (1937).

(20) F. Ehrlich, *Chem.-Ztg.*, 41, 197 (1917); R. Ripa, "Die Pektinstoffe," *Serger & Hempel*, Braunschweig, Germany (1925).

primitive pectin was that portion of pectin which was insoluble in cold water and which was converted into water-soluble "hydratopectin" by the action of hot water under pressure. He showed that this "hydratopectin" consisted of a water-soluble araban and a "pectinic acid" which gave insoluble calcium and magnesium salts.<sup>21</sup> F. Ehrlich considered that this "pectinic acid" still contained combined araban and galactan. On mild acid hydrolysis it gave methyl alcohol, D-galacturonic acid, D-galactose, L-arabinose and a polygalacturonic acid. Different varieties of polygalacturonic acids were isolated which this author regarded as polymers of a cyclic tetragalacturonic acid—the so-called pectolic acid—which he thought to be the unit on which the structure of pectin was based.<sup>22</sup> It was considered that this cyclic pectolic acid was converted by hydrolysis into the open chain pectolactonic acid (see accompanying scheme for summary; Fig. 1).

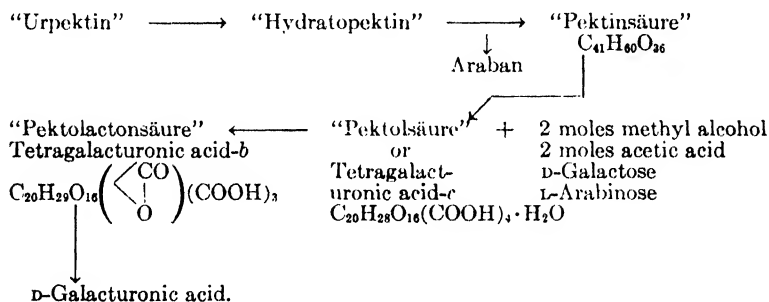


FIG. 1.—F. Ehrlich's scheme for the hydrolysis products of "Urpektin."

F. Ehrlich examined pectins from many sources and showed that the products were very similar in properties and reactions, although he preferred to believe that this similarity was merely coincidental and that pectins from different sources were chemical entities and not simply differently proportioned mixtures of the same polysaccharides.<sup>23</sup> Nanji, Paton and Ling<sup>16</sup> also were of the opinion that the pectin molecule was one of low molecular weight and after considering the analytical data obtained from a whole series of pectin samples, they suggested that the properties of pectic acid could best be explained on the basis of a six-membered ring containing D-galactose, L-arabinose, and D-galacturonic acid residues (see Fig. 2).

More recently, the chemical structure of the components of pectic materials has been investigated by the present writers, whose approach

(21) F. Ehrlich and F. Schubert, *Biochem. Z.*, 203, 343 (1928).

(22) A. Kosmahly, Dissertation (1929); Springer, Berlin (1929).

(23) F. Ehrlich, *Biochem. Z.*, 250, 525 (1932).

to the subject has been made from a rather different point of view.<sup>2</sup> Consideration of the polysaccharides cellulose and xylan, which contain respectively D-glucose and D-xylose residues, shows that they are closely associated with one another in nature. Furthermore, it has been demon-

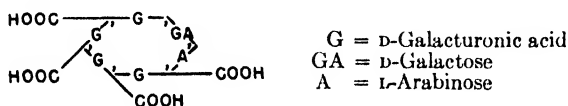


FIG. 2.—The Nanji, Paton and Ling formula for pectic acid.

strated that the ring structure and the linkage of the D-xylose and D-glucose units in the respective polysaccharides are in agreement with the hypothesis that the D-xylose residues in xylan could have been derived from the D-glucose residues in cellulose by oxidation of the primary alcoholic groups to carboxyl groups, with subsequent decarboxylation.<sup>2, 24</sup>

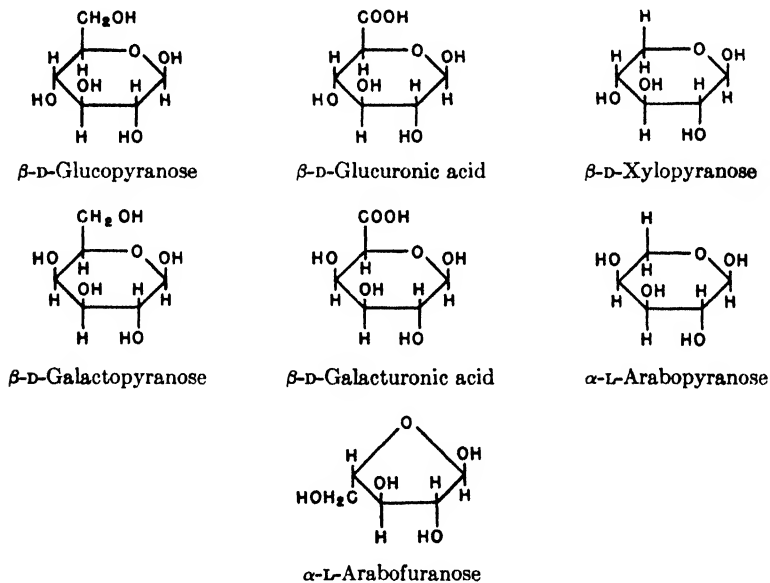


FIG. 3.—Relationship between D-glucose and D-xylose, D-galactose and L-arabinose.

In view, however, of the most recent work on the structure of xylan,<sup>24, 25</sup> which has been shown to contain combined L-arabinofuranose residues in

(24) W. N. Haworth, E. L. Hirst and Elsie Oliver, *J. Chem. Soc.*, 1917 (1934).

(25) W. N. Haworth, *Monatsh.*, 69, 314 (1936).

terminal positions in the chain, this hypothesis cannot be regarded as very probable; nevertheless, the very striking stereochemical relationships subsisting among the residues of D-galactose, D-galacturonic acid, and L-arabinose which are closely associated in pectic materials (see Fig. 3) appeared to render it worth while to pursue further enquiries as to whether the L-arabinose residues in pectin could be regarded as derived from the D-galactose residues by a process of oxidation followed by decarboxylation. It seemed clear that if separation of the araban, galactan, and polygalacturonic acid portions of pectin could be achieved and the molecular constitution of the respective polysaccharides determined, then further evidence would be available regarding the significance of the association of these three polysaccharides with one another. The results of the work will be considered at appropriate stages later in this article, and in the meantime we proceed to discuss in somewhat greater detail the evidence concerning the chemical structure of pectic acid, araban and galactan.

### III. PECTIC ACID

The constitution of the pectic acid fraction of pectin has been investigated by many workers. Suarez<sup>26</sup> and Ehrlich<sup>20</sup> were the first to show that pectic acid gave D-galacturonic acid after hydrolysis with acid or with an enzyme from the mold *Penicillium ehrlichii*. The latter method has become of considerable industrial importance in preparing fruit drinks and wines free from haze due to precipitated pectic acid.<sup>27, 28</sup> The preparation of a pectic acid free from adsorbed galactan and araban by physical means is very difficult to achieve owing to the colloidal properties of the system. Separation by chemical methods is hindered by the instability (although in varying degrees) of all three polysaccharides toward acids and is further complicated by the instability of pectic acid toward alkali. The latter reaction may be connected with the tendency of D-galacturonic acid to enolize in the presence of alkali with the formation of 5-keto-L-galactonic acid (see Fig. 5). Although pectic acid gives insoluble calcium and copper salts, this property cannot be used to isolate galactan and araban free from pectic acid unless the latter is present to a small extent only. The removal of araban is a comparatively easy operation but the isolation of a pectic acid free from both galactan and araban has only been achieved by starting from a pectin initially low in galactan or by the use of boiling, methyl alcoholic hy-

(26) L. Suarez, *Chem.-Ztg.*, **41**, 87 (1917).

(27) E. A. Oxford, *Nature*, **154**, 271 (1944).

(28) E. Rietz and W. D. Mackay, *J. Am. Chem. Soc.*, **65**, 1242 (1943).



drogen chloride, which preferentially destroys araban and galactan but at the same time degrades the pectic acid.<sup>18</sup> The latter method was used by Baur and Link<sup>18</sup> to prepare from several pectic acids polygalacturonides containing 8 to 10 uronic acid residues. Henglein and Schneider<sup>29</sup> investigated the constitution of pectic acid from a different point of view; they nitrated it under controlled conditions and examined the physical properties of solutions of the resultant nitropectin. Their

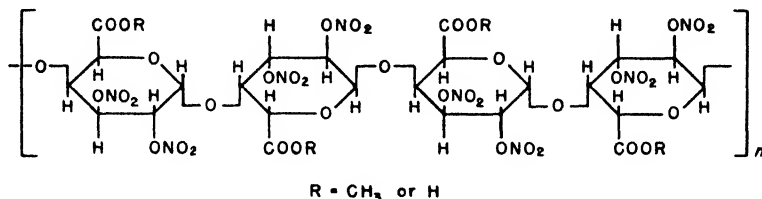


FIG. 4.—Nitropectin (after G. G. Schneider and U. Fritsch).

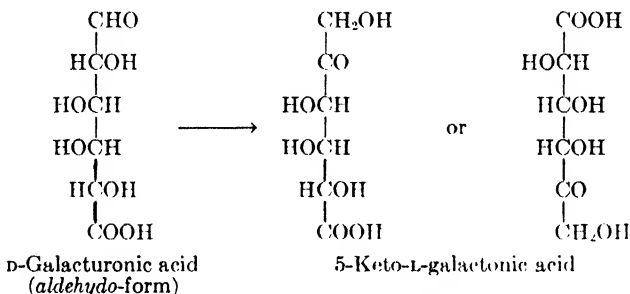


FIG. 5.—Alkaline rearrangement of D-galacturonic acid.

results led them to the conclusion that pectic acid was a substance of high molecular weight and that it consisted exclusively of condensed D-galacturonic acid units—they advocated, in common with Baur and Link<sup>18</sup>, a 1,4-glycosidic linkage as part of the pectic acid molecule (see Figs. 6 and 9). The first direct chemical evidence in favor of this was put forward by Levene and Kreider,<sup>30</sup> who oxidized a pectic acid with per-iodic acid and obtained D(*levo*)-tartaric acid after acid hydrolysis, followed by bromine oxidation of the product (see Fig. 7). Isolation of this product, however, does not decide whether the D-galacturonic acid is in the furanose form and linked 1,5 or in the pyranose form and linked 1,4. Direct chemical evidence in favor of either of these hypotheses is wanting but the extraordinary stability of pectic acid to acid hydrolysis suggests

(29) F. A. Henglein and G. Schneider, *Ber.*, 69, 309 (1936).

(30) P. A. Levene and L. C. Kreider, *J. Biol. Chem.*, 120, 591 (1937).

that the uronic acid is present as the pyranose form and that the linkage is therefore 1,4 (see Fig. 7). Further evidence that the hydroxyl groups on carbon atoms C<sub>2</sub> and C<sub>3</sub> are free and that the hydroxyl groups on carbon atoms C<sub>4</sub> and C<sub>5</sub> are masked by glycoside and ring formation, respectively, has been given by Beaven and J. K. N. Jones and by F. Smith.<sup>31, 32</sup>

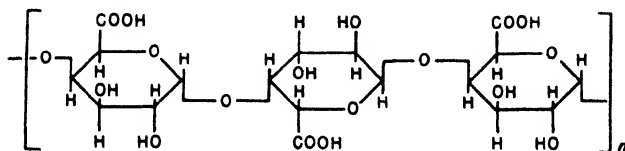


FIG. 6.—Possible formula for degraded pectic acid.

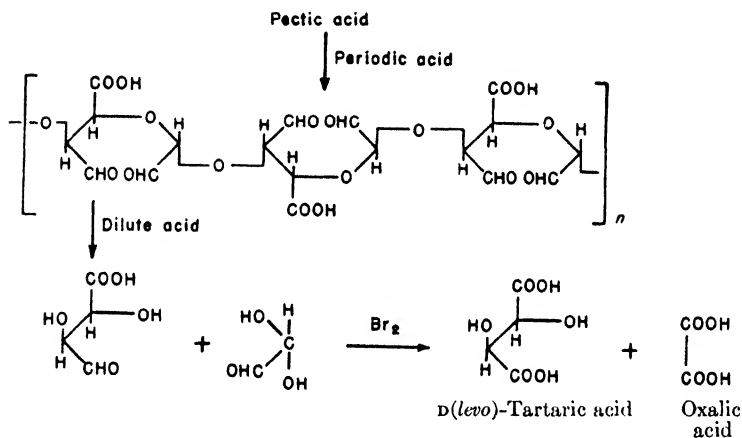


FIG. 7.—Oxidation of pectic acid with per-iodic acid.

The present writers and their collaborators have examined degraded polygalacturonic acids derived from the pectic acids present in the apple, strawberry, peanut, and others.<sup>2</sup> These degraded products were used because, initially, too many experimental difficulties were encountered in attempts to methylate the undegraded pectic acid. The polygalacturonic acids were methylated by heating their thallium salts with methyl iodide. Methanolysis of the methylated polyesters gave the methyl ester of methyl 2,3-dimethyl-D-galacturonoside as a mixture of its pyranose and furanose forms (see Fig. 8). This product was identified after oxidation to the dimethyl ether of L(dextro)-tartaric acid. In a

(31) G. H. Beaven and J. K. N. Jones, *Chemistry & Industry*, 363 (1939).

(32) F. Smith, *Chemistry & Industry*, 363 (1939).

similar manner, Luckett and F. Smith<sup>33</sup> isolated the methyl ester of methyl 2,3-dimethyl-D-galacturonoside from a methylated polygalacturonic acid derived from citrus pectin. Neither of these groups of workers succeeded in isolating any methyl 2,3,4-trimethyl-D-galacturonoside and thereby obtaining some indication of the chain length of the polyester. The reason for this absence of end group is unexplained. The evidence so far indicates that degraded polygalacturonides practically identical in chemical and physical properties may be isolated from several different pectic acids and that there is strong evidence in favor of pectic acids from several plant sources being of very similar,

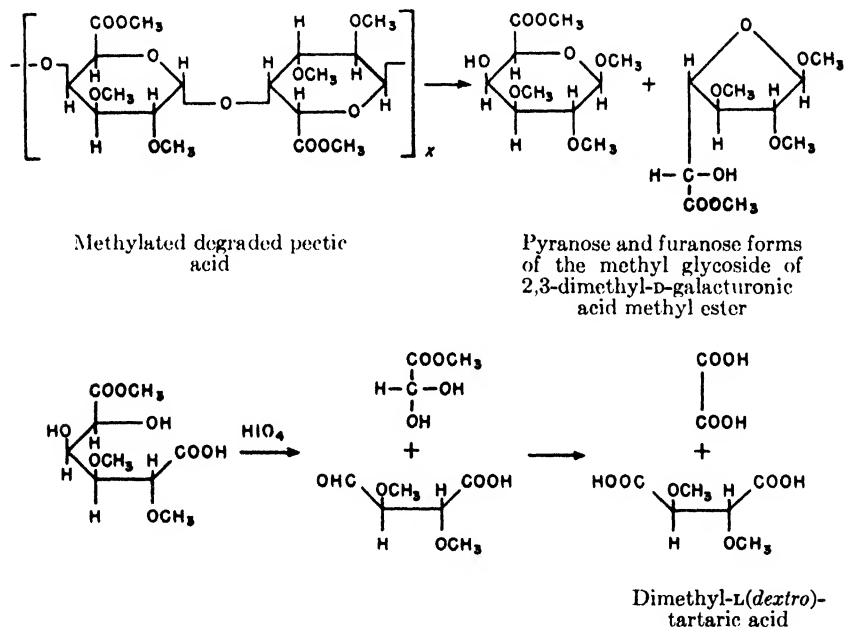
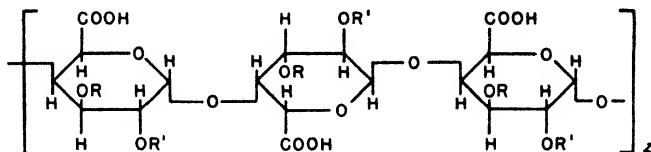


FIG. 8.—Constitution of methylated degraded pectic acid.

if not identical constitution. This pectic acid, free from araban and galactan, has  $[\alpha]_{D^{20}}$  ca.  $+280^\circ$  in water. It is uncertain whether it consists entirely of D-galacturonic acid residues or whether small amounts of other sugars may be present. Since only degraded pectic acids have so far been examined, there remains the possibility that undegraded pectin may consist of a main chain of D-galacturonic acid residues linked through carbon atoms 1 and 4, and that side chains of D-galacturonic acid units in the pyranose or furanose form may be attached to this main

(33) Sybil P. Luckett and F. Smith, *J. Chem. Soc.*, 1106 (1940).

portion of the molecule. Hydrolysis by the method of Link<sup>18</sup> would then result in elimination of the more easily hydrolyzed side chains and leave a degraded pectic acid in which the D-galacturonic acid residues are pyranose and linked through positions 1 and 4 (see Fig. 9).



Side chains of D-galacturonic acid residues in the pyranose or furanose forms may be attached to one or both of the positions R and R'.

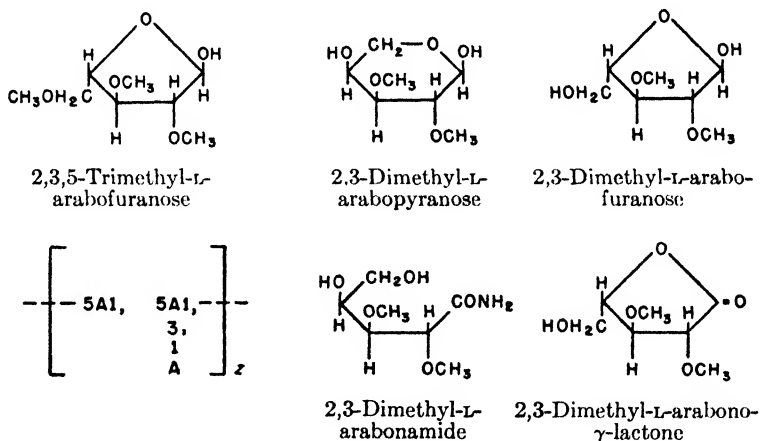
FIG. 9.—Possible formula for undegraded pectic acid.

#### IV. ARABAN

In the course of experiments by which it was hoped to gain further insight into the phytochemical relationships of the hexose and pentose sugar residues in the hemicelluloses (see Fig. 3), the present authors isolated a polysaccharide from the peanut<sup>34</sup> which showed all the properties of crude pectin. By careful fractionation of this product, araban was obtained in a condition practically free from other polysaccharides, and by fractionation of the methylated derivative prepared from the crude polysaccharides, separation of a nearly pure methylated araban was effected. Hydrolysis of this product demonstrated that the structure of the methylated polysaccharide was very different from that of the pectic acid and the galactan with which it was associated in the peanut. The unmethylated araban, which had a negative rotation, was hydrolyzed by dilute acid with great ease and it behaved in all respects as a typical derivative of L-arabofuranose, giving on hydrolysis, L-arabinose in approximately 95% yield. The remaining 5% sugars have not yet been identified and it is still possible that some sugar other than L-arabinose may be present. A quantitative examination of the products formed from the methanolysis of the methylated araban proved that the polysaccharide was of the branched chain type since approximately one-third of the product was methyl trimethyl-L-arabofuranoside, one-third methyl 2,3-dimethyl-L-arabinoside, and one-third methyl monomethyl-L-arabinoside. The dimethyl-L-arabinose residue might have existed in the polysaccharide either as a furanose or pyranose form, but the high rate of hydrolysis of the araban in dilute acid was strong evidence that all of the pentose molecules were present in the furanose forms. In one case

(34) E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 502 (1938).

the methylated araban gave some L-arabinose on hydrolysis, but whether this is an integral portion of the molecule or is derived from incompletely methylated polysaccharide has not yet been ascertained. One of the many problems which still await solution concerns the identity of the monomethyl-L-arabinose, the structure of which has not been ascertained with as great a degree of certainty as for the trimethyl-L-arabofuranose and the 2,3-dimethyl-L-arabinose. It has not been possible to isolate a characteristic crystalline derivative, and the complete identity of this material has not yet been decided but there are indications that the material is 2-methyl-L-arabinose. The evidence so far available is therefore not sufficiently detailed to enable a unique structure to be assigned to the repeating units of the araban molecule, but it is clear that a branched chain structure must be present, and the formula given in Fig. 10 indicates the general type of structure which is present. Apart from the lack of precision due to the uncertainty concerning the identity of the monomethyl-L-arabinose fraction, there are obvious variants of this that would explain the experimental facts, and further work will be required before a definite choice can be made.



A = α-L-arabofuranose residues united to other residues through the numbered C-atoms.

FIG. 10.—Araban from pectin (possible formula).

The present authors and their collaborators have also investigated arabans derived from apple and citrus pectins and here again it is to be noted that the arabans are associated in nature with galactan and pectic acid. The methylated derivatives of all these arabans have very similar properties and on hydrolysis give approximately the same proportions of 2,3,5-trimethyl-L-arabofuranose, 2,3-dimethyl-L-arabinose and

monomethyl-L-arabinose. It is highly probable, therefore, that these arabans are structurally identical with one another. In this connection it may be noted that both Ehrlich and Schubert<sup>21</sup> and Gaponenkov<sup>35</sup> have investigated arabans obtained from various sources of pectin and have recorded physical constants with which the data obtained in the recent work are in reasonable agreement. It is likely, therefore, that all the samples of araban possessed the same type of constitution. The evidence so far available suggests, therefore, that pectic materials from a wide variety of sources contain the same araban, which is a branched chain polysaccharide built up mainly, if not entirely, of L-arabofuranose residues.

The only other araban which has hitherto been examined from the point of view of its chemical structure is the one present in gum tragacanth.<sup>36</sup> This araban has properties similar to those of the araban just described, but in addition to L-arabinose it contains a small amount of D-galactose.

We may return now to the polysaccharides present in the peanut for a brief consideration of the relationship of the other components present in the pectic materials to the araban constituent. All the evidence indicates that the pectic acid portion of the peanut is identical with normal pectic acid and, as was indicated in the previous section, this material, which is very stable to acid hydrolysis and possesses a high positive rotation contains a main chain which is built up of D-galacturonic acid residues of the pyranose type. If, therefore, the araban associated with the pectic acid had been derived directly from the pectic acid by decarboxylation without intermediate hydrolysis of the polygalacturonide, the sugar residues in the araban should also be in the pyranose form. The experimental evidence shows clearly, however, that the arabinose residues in araban are furanose in type and it follows that any hypothesis concerning the direct conversion of pectic acid into the araban by decarboxylation is untenable.

These observations have a bearing also on certain claims<sup>37</sup> which have been made concerning a supposed increase in the pentosan content when pectin is heated in alkaline solution. It is obvious that any increase in the proportion of pentosan in the product cannot be due to decarboxylation of the pectic acid molecule with the formation of the araban. The

(35) T. K. Gaponenkov, *Kolloid Zhur.*, **2**, 561 (1936).

(36) S. P. James and F. Smith, *J. Chem. Soc.*, 749 (1945); B. Tollens and H. Elsner, "Kurztes Handbuch der Kohlenhydrate," 4th Edition, p. 306. J. A. Barth, Leipzig (1935).

(37) E. J. Candlin and S. B. Schryver, *Proc. Roy. Soc. (London)*, **B**, **103**, 365 (1928); F. J. Linggood, *Biochem. J.*, **24**, 262 (1930).

observations are probably to be explained by the sensitivity of pectic acid toward alkali. During the treatment some of the pectic acid is destroyed and there is a resultant concentration in the mixture of the more resistant araban.

#### V. GALACTAN

By selection of an appropriate pectin in which the desired component is present in suitable proportion, it is possible without undue difficulty to separate either the pectic acid portion or the araban in a sufficient state of purity for structural investigation. On the other hand, it has been more difficult to isolate the galactan component. In the course of some investigations of the polysaccharides present in the peanut, Miyama<sup>38</sup> reported the isolation of material containing L-arabinose, D-galactose and uronic acid residues. He considered, however, that the uronic acid present in his material was an artifact which had been derived from the galactan portion by alkaline oxidation during the procedure he had used for extraction, a conclusion which is not supported by later evidence. The same author concluded that the main polysaccharide was a galacto-araban, the L-arabinose residues of which were present in the pyranose form. In the latter respect, Miyama's conclusion is based on a misinterpretation of the evidence and as has been shown above, it is clear that the L-arabinose residues are present in the furanose condition. The possibility envisaged by Miyama that pectic materials from this source might contain a mixed polysaccharide containing both D-galactose and L-arabinose residues was of such interest that the present writers paid particular attention to this aspect of the problem. They have not, however, succeeded in isolating such a mixed polysaccharide and the evidence as it stands at present points rather to the existence in pectic materials of a definite araban mixed with a definite galactan. The peanut polysaccharide mixture was found to be most unpromising in respect to the possible isolation of the galactan portion and although clear indications of the presence of galactan were obtained, up to the present all attempts to isolate this in a pure condition have been unsuccessful.

So far as we are aware, the galactan associated with pectin has hitherto been isolated in a reasonable degree of purity in one instance only,<sup>2</sup> namely from the seeds of *Lupinus albus*. This source contains a comparatively high proportion of the galactan component ( $[\alpha]_D + 35^\circ$  in water) and the isolation of the galactan is thus facilitated. The araban was partially removed by repeated reprecipitation from aqueous solution

(38) R. Miyama, *J. Dept. Agr. Kyushu Imp. Univ.*, 4, 195 (1936).

by addition of alcohol, whilst the major portion of the pectic acid was separated in the form of its insoluble calcium salt. The crude galactan thus obtained was then subjected to methylation and a pure methylated galactan was eventually obtained by separating the methylated araban from the crude methyl derivative with ether, in which methylated galactan is insoluble. The methylated galactan ( $[\alpha]_D^{20} = -16^\circ$  in  $\text{CHCl}_3$ ) was very resistant to acids and required long boiling with 5% methyl alcoholic hydrogen chloride to effect hydrolysis. Examination of the

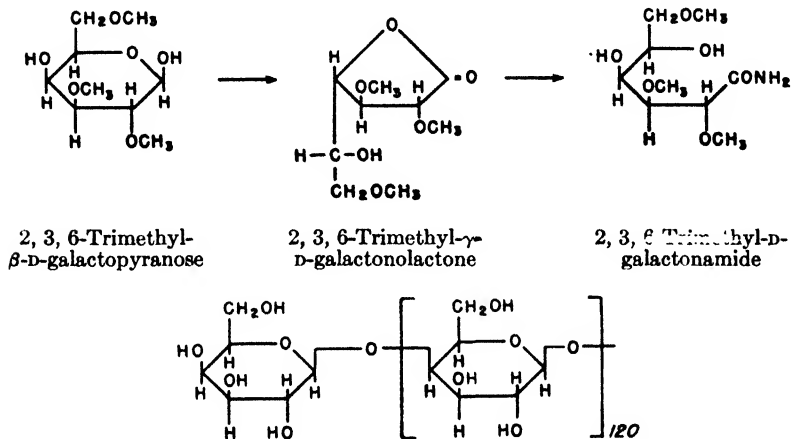


FIG. 11.—Galactan from pectin.

products of methanolysis showed the presence of methyl 2,3,6-trimethyl-D-galactoside, identified after hydrolysis and oxidation to 2,3,6-trimethyl-D-galactonolactone, along with a small amount of methyl 2,3,4,6-tetramethyl-D-galactoside and some unidentified methyl dimethyl-hexoside which may have been a methylated D-galactose derivative. The amount of methyl 2,3,4,6-tetramethyl-D-galactoside indicated a repeating unit of about 120 monomeric units in the galactan molecule. The low negative rotation of the methylated polysaccharide, taken in conjunction with the relative stability of the polysaccharide to methyl alcoholic hydrogen chloride and the isolation of some methyl 2,3,4,6-tetramethyl-D-galactoside from the products of hydrolysis, indicates very strongly that the galactan consists of a chain of some 120 D-galactopyranose units united to one another by 1,4- $\beta$ -links. The presence of small amounts of other sugars cannot be excluded at this stage (see Fig. 11).

Only one other naturally occurring galactan has been examined in any great detail; this is the  $\epsilon$ -galactan of the larch.<sup>40</sup> It contains D-galacto-

(40) A. W. Schorger and D. F. Smith, *J. Ind. Eng. Chem.*, **8**, 494 (1916).



pyranose and L-arabofuranose units and is a branched chain polysaccharide,<sup>2,41,42</sup> very different from the galactan isolated from pectin (see Fig. 12).

Although pectic materials are known to be present in wood, it is not clear whether this  $\epsilon$ -galactan is a component of wood pectin, and in view of the wide difference in structure between this galactan and the one associated with pectin in lupin seeds, further investigations of this point will be awaited with special interest.

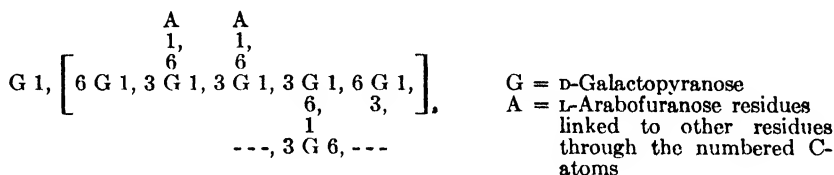


FIG. 12.—Repeating unit of  $\epsilon$ -Galactan (possible formula) after E. V. White.

Although the ring structures of the sugar residues in both pectic acid and in the galactan associated with it in pectic materials are of the pyranose type, the presence of the  $\alpha$ -glycosidic group in the one and the  $\beta$ -glycosidic group in the other rules out immediately any possibility of direct conversion of the galactan into pectic acid by enzymic oxidation of the terminal  $CH_2OH$  groups. On the other hand, the close stereochemical relationship between D-galactose, D-galacturonic acid and L-arabinose would appear to be significant in indicating the derivation of the pentose sugar from the corresponding hexose by a transformation involving the loss of the terminal carbon atom. Such a view would be in accordance with the bulk of evidence at present available concerning the synthesis of carbohydrate materials in nature, where the first recognizable sugars appear to be hexoses. Nevertheless, the evidence which has been cited in the foregoing summary of the structural chemistry of pectic materials indicates that neither the pectic acid nor the araban components can be derived directly from galactan of the type which has been isolated from pectic materials. To account for such a transformation by oxidation and decarboxylation the simplest hypothesis at the moment is to suppose that the galactan is hydrolyzed in the plant to the free sugar, and the resultant D-galacturonic acid is then converted to pectic acid. Similarly, it must be supposed on the basis of this hypothesis that D-galacturonic acid produced either from pectic acid or by oxidation of D-galactose is decarboxylated, after which the resultant

(41) E. L. Hirst, J. K. N. Jones and W. G. Campbell, *Nature*, **147**, 25 (1941).

(42) E. V. White, *J. Am. Chem. Soc.*, **63**, 2871 (1941); **64**, 302, 1507, 2838 (1942).

L-arabinose molecules combine together in the furanose form to produce an araban. Since enzymes capable of hydrolyzing pectic acid to D-galacturonic acid are known to be present in certain plant juices, and since the comparatively low temperature and slightly acid condition of plant materials would tend to favor the transformation of arabinose into the furanose form, the occurrence of the furanose structure in the polysaccharide derived from arabinose is not altogether unexpected.

Alternatively, if we suppose that the hexose polysaccharide is produced first and is then transformed into pectic acid and araban, at least two types of galactan, other than the one already isolated from pectic materials, would be required to account for the types of structure present respectively in pectic acid and araban. This would seem to be somewhat unlikely since, on this view, one would expect to encounter other types of pectic acid and araban, whereas on the evidence now available the pectic acid isolated from all samples of pectic materials examined appears to be the same, and a similar conclusion applies in the case of the araban.

It is clear, however, that the extensive problems concerning phytochemical relationships which have been raised as the result of the work summarized in this review cannot be resolved in the present state of knowledge. So far, in spite of the considerable attention given by many workers in this difficult field, little more than a preliminary survey of the possibilities has been achieved. Further investigations, which will almost certainly require novel methods of approach, will be necessary in order to decide unequivocally the structural formulas of the components of pectic materials, and only when such further knowledge is available will it be feasible to reach definite conclusions regarding their phytochemical relationships.



# THE POLYFRUCTOSANS AND DIFRUCTOSE ANHYDRIDES

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## I. POLYFRUCTOSANS

Many plants store carbohydrates in their tubers in the form of polysaccharides which upon acid hydrolysis yield D-fructose as the main product. These polysaccharides differ from one another in the size of the molecule as well as in the position of the linkages between the D-fructose residues. Polyfructosans have also been prepared by enzymic action on sucrose.

Progress in the study of this field has been impeded by the difficulties in obtaining pure compounds. Since the polysaccharides as a rule have

not been isolated in crystalline form, the criterion used in determining the homogeneity of a product has been an important factor in determining the results in any given investigation.

### 1. *Inulin*

Inulin was first isolated and, indeed, has received by far the most attention. It was discovered in 1804 by Rose<sup>1</sup> who separated it from the extract of artichoke tubers. The name inulin was first used by Thomson<sup>2</sup> in 1811.

Inulin can be prepared most satisfactorily from dahlia tubers, in somewhat lower yield from chicory, and in much lower yield from Jerusalem artichokes. The most suitable time for the extraction is early autumn, but in the case of dahlias the large clumps of tubers are usually divided for replanting in the late spring and the "blind" tubers that are unsuitable for planting are then available for inulin extraction. Even as late as early summer, inulin may be obtained from healthy tubers. The following procedure for preparing inulin is given in the National Bureau of Standards Circular C440.

*Preparation of Inulin.* Comminute the tubers or roots in a food chopper or similar appliance and express the juice with a tincture press, using, if necessary, a small portion of water to complete the extraction. Heat the juice to 60-70° and add milk of lime to about pH 8. Filter and adjust the pH to 7 with oxalic acid. Heat to 70-80°, add activated carbon and filter. Allow the filtrate to stand quiescent overnight, during which time the inulin separates in the form of small spheroids. The yield may be increased by freezing the solution and allowing it to thaw at a low temperature. Filter and wash the inulin with abundant quantities of cold water.

The moist cake of inulin has at this point a dry substance content of 35-40%. It can be purified by dissolving in hot water to a concentration of about 15-18%, treating with carbon, filtering, and allowing to separate again by chilling.

For many purposes it is satisfactory to spread the moist inulin cake on a glass plate and allow it to dry in air. When dried this way, it takes on the form of hard horn-like masses which can be pulverized readily. The air-dried substance contains about 10% of water of hydration and a small quantity of inorganic impurities. The latter can be diminished to negligible proportions by repeating the process of solution and chilling, or by electrodialysis.

To avoid the formation of horn-like masses, the moist inulin cake can be washed with 95% alcohol, followed by absolute alcohol. Inulin, however, has a tendency to retain alcohol.<sup>3</sup>

(1) V. Rose, *Neues Allgem. J. Chem. (Gehlens)*, **3**, 217 (1804).

(2) T. Thomson, "A System of Chemistry," **4**, 65, from 5th London edition, Abraham Small, Philadelphia (1818).

(3) E. Berner, *Ber.*, **64**, 842 (1931).

A procedure that is described by Tollens and Elsner<sup>4</sup> is probably suitable for materials less pure than fresh dahlia juices. The juice is expressed in the presence of calcium carbonate, fermented at 25° with baker's yeast, defecated with lead acetate and filtered. After removal of the excess lead with hydrogen sulfide, the filtrate is frozen and thawed to cause the separation of inulin.

Inulin crystallizes in the form of doubly refracting spherocrystals, the crystalline structure of which is indicated by x-ray patterns.<sup>5,6</sup> In aqueous solution inulin has a specific rotation  $[\alpha]_{D^{20}} = -39$  to  $-40^\circ$  referred to the anhydrous substance.

If heated in glycerol,<sup>7-9</sup> or ethylene glycol, or even in water solution and precipitated from these solutions by alcohol, inulin separates in a form soluble in cold water. This soluble form is also produced by the action of carbon dioxide.<sup>10</sup>

The solubility<sup>11</sup> of inulin in water varies greatly with temperature and mode of preparation. Conflicting reports are to be found as to the exact amount that is held in solution at a given temperature. Yanovsky and Kingsbury<sup>12</sup> have determined the solubility of inulin prepared from dahlias and from chicory. They found a marked difference in the solubility of the two products. When recrystallized from water the solubility of inulin was considerably less than when precipitated by alcohol. Upon standing in the presence of water, the more soluble  $\beta$ -modification gradually changed into the less soluble  $\alpha$ -form. It seems quite possible that the lack of agreement among different investigators relative to the physical properties of inulin is due to variations in the method of isolation and the degree of purification.

Jackson and McDonald<sup>13</sup> analyzed samples of inulin from various sources. They obtained a uniform product by recrystallization from water, irrespective of the source. Since they determined total solids by refractive index and density measurements on the hydrolyzed inulin, they were not concerned with its crystalline form nor with its moisture content.

(4) B. Tollens and H. Elsner, "Kurztes Handbuch der Kohlenhydrate." J. A. Barth, Leipzig (1935).

(5) J. R. Katz and A. Weidinger, *Rec. trav. chim.*, **50**, 1133 (1931).

(6) J. R. Katz and J. C. Derksen, *Rec. trav. chim.*, **50**, 248 (1931).

(7) H. Pringsheim, J. Reilly and P. P. Donovan, *Ber.*, **62**, 2378 (1929).

(8) H. Pringsheim and J. Reilly, *Ber.*, **63**, 2636 (1930).

(9) H. Vogel, *Ber.*, **62**, 2980 (1929).

(10) H. D. K. Drew and W. N. Haworth, *J. Chem. Soc.*, 2690 (1928).

(11) E. O. von Lippmann, "Chemie der Zuckerarten," p. 797. Vieweg, Braunschweig (1904).

(12) E. Yanovsky and R. M. Kingsbury, *J. Am. Chem. Soc.*, **55**, 3658 (1933).

(13) R. F. Jackson and Emma J. McDonald, *Bur. Standards J. Research*, **5**, 1151 (1930).

The presence of D-glucose in the hydrolytic products of inulin has been noted by many investigators. Tanret<sup>14</sup> showed that the rotatory power of acid-hydrolyzed inulin corresponds not to pure D-fructose but to a mixture of D-fructose and D-glucose in the ratio 12:1. Pringsheim and Reilly<sup>8</sup> hydrolyzed inulin by means of 0.05 N sulfuric acid and analyzed the products of hydrolysis by means of Bertrand's method for total reducing sugar and by Willstätter's method for aldoses. After correcting for the effect of D-fructose upon the aldose analysis, they found 5% D-glucose. Schlubach and Elsner<sup>15</sup> by means of acetolysis of inulin acetate were able to identify pentaacetyl- $\alpha$ -D-glucose as one of the final products. Jackson and Goergen<sup>16</sup> and Jackson and McDonald<sup>13</sup> determined total reducing sugar by Lane and Eynon's method and D-fructose by Nyns' method; the difference they assumed to be D-glucose. Their results indicate that when inulin is hydrolyzed by acid, an average of 3.3% D-glucose is formed, the extremes being 1.9% and 4.4%. It is interesting to note the agreement in the rotation (D-line, room temperature, water) of the acid hydrolysis product as reported by different investigators: Tanret,<sup>14</sup> - 81.3°; Schlubach and Knoop,<sup>17</sup> - 79.1°; Jackson and McDonald,<sup>13</sup> - 79.9°.

Jackson and Goergen<sup>16</sup> showed that the product of acid hydrolysis contained, along with D-fructose and D-glucose, about 5% of nonreducing difructoses. Whether these difructose anhydrides are an integral part of the inulin molecule or whether they are formed during the acid hydrolysis has not been conclusively shown. It seems most probable that the latter is true. This nonreducing residue has a specific rotation of + 56° (D-line, 20°, water) and hence rotation measurements do not differentiate between it and D-glucose. Tanret's estimate of D-glucose does not take these products into consideration.

By enzymic hydrolysis with inulase prepared from *Aspergillus niger*, Pringsheim and Ohlmeyer<sup>18,19</sup> found 1.5% D-glucose in the inulin hydrolyzate. Adams, Richtmyer and Hudson<sup>20</sup> report the presence of 1.7% D-glucose when inulin is hydrolyzed by baker's yeast invertase preparation. They conclude that "if glucose is not an integral part of the inulin molecule it must be an integral part of an associated molecule

(14) C. Tanret, *Bull. soc. chim.*, [3] 9, 233 (1893).

(15) H. H. Schlubach and H. Elsner, *Ber.*, 62, 1493 (1929).

(16) R. F. Jackson and Sylvia M. Goergen, *Bur. Standards J. Research*, 3, 27 (1929).

(17) H. H. Schlubach and H. Knoop, *Ann.*, 497, 229 (1932).

(18) H. Pringsheim and P. Ohlmeyer, *Ber.*, 65, 1242 (1932).

(19) P. Ohlmeyer and H. Pringsheim, *Ber.*, 66, 1292 (1933).

(20) Mildred Adams, N. K. Richtmyer and C. S. Hudson, *J. Am. Chem. Soc.*, 65, 1369 (1943).

which is hydrolyzed at about the same rate as inulin." These authors used five yeast invertase preparations in a study of the enzymes involved in the hydrolysis of sucrose, raffinose, and inulin. The pH of maximum activity for inulin hydrolysis was found to be 3.5 to 3.7 as compared to pH 5.0 to 5.5 for sucrose and raffinose. The authors thus believe that the enzymic hydrolysis of inulin may be brought about by a specific inulase rather than by the  $\beta$ -D-fructofuranosidase as postulated by Weidenhagen.<sup>21</sup>

Pigman,<sup>22</sup> in an investigation of the inulin-hydrolyzing enzymes in commercial enzyme preparations, has noted the same pH for optimum activity. He concludes that the inulases and invertases are different enzymes or that they represent a class of enzymes in which the individual members vary according to source.

Boiling point, freezing point, osmotic pressure, vapor pressure, and viscosity determinations have been used in studying the molecular weight of inulin. Molecular weight values varying from 312 to 6,000 have thus been obtained. The difficulty of preparing a pure ash-free sample and the apparent decomposition of inulin in hot solution probably account for a large part of this variation.

Haworth, Hirst and Percival<sup>23</sup> methylated inulin by first acetylating and subsequently converting the acetate to the methyl derivative by means of dimethyl sulfate and sodium hydroxide in acetone solution, a procedure described by Haworth and Streight.<sup>24</sup> They hydrolyzed trimethylinulin and quantitatively determined the amounts of trimethyl-D-fructose and tetramethyl-D-fructose formed. There was obtained 3.7% 1,3,4,6-tetramethyl-D-fructose. Since this was assumed to result from a terminal D-fructose residue, the authors concluded that inulin is composed of about 30 D-fructose residues and has a molecular weight of about 5,000. These authors believe that their inability to obtain a non-reducing inulin sample is a strong indication that one terminal D-fructose has a free reducing group. However, the apparent breakdown of inulin in boiling water, as signified by a decrease in molecular weight, should not be overlooked when interpreting the results of reducing sugar analysis.

The detailed procedure for the preparation of inulin acetate and conversion to its methyl derivative by the method of Haworth and Streight<sup>24</sup> is as follows.

(21) R. Weidenhagen. *Z. Ver. deut. Zucker-Ind.*, **79**, 115 (1929); *Fermentforschung*, **11**, 155 (1930); *Ergeb. Enzymforsch.*, **1**, 168 (1932); C. Oppenheimer, "Die Fermente und ihre Wirkungen," Suppl. Bd. 1, Dr. W. Junk Verlag, Den Haag (1936), p. 191; Nord-Weidenhagen, "Handbuch der Enzymologie," p. 512, Akademische Verlagsgesellschaft, Leipzig (1940).

(22) W. W. Pigman, *J. Research Natl. Bur. Standards*, **30**, 159 (1943).

(23) W. N. Haworth, E. L. Hirst and E. G. V. Percival, *J. Chem. Soc.*, 2384 (1932).

(24) W. N. Haworth and H. R. L. Streight, *Helv. Chim. Acta*, **15**, 609 (1932).



*Inulin Acetate.* Stir 100 g. of inulin in 1,000 ml. of pyridine at 80° for forty-five minutes. Cool, while continuing the stirring, and to the clear solution add dropwise 180 ml. of acetic anhydride. After six hours more of stirring, pour the clear solution into 10 liters of water. The inulin acetate separates as a white solid. Filter and wash repeatedly with distilled water to remove the pyridine and acetic anhydride. Purify the dried crude product by dissolving in ten times its weight of hot methyl alcohol and filtering. Inulin acetate separates from the cold solution as a fine white powder;  $[\alpha]_D^{20} = -34^\circ$  ( $c = 1.5$ , chloroform),  $[\alpha]_D^{20} = -43^\circ$  ( $c = 1.8$ , acetic acid).

Molecular weight determinations by Rast's<sup>25</sup> method gave a value of 6,300. The acetate prepared by reacting at 40° for a much longer period of time had a molecular weight<sup>24</sup> of 3,000 (molecular weight determined as above), thus indicating that prolonged heating caused degradation of the molecule. Triacetylinulin prepared at 140° by the method of Pringsheim and Aronowsky<sup>26</sup> resulted in much lower yields. It is probable that degradation products soluble in methyl alcohol resulted.

*Trimethylinulin.* Prepare a solution containing 12 g. of triacetylinulin and 250 ml. of acetone in a 2-liter flask fitted with a mechanical stirrer. While keeping the temperature at 55°, gradually add 120 ml. of dimethyl sulfate and 320 ml. of 30% sodium hydroxide (add 1/10 of the reagents every 10 minutes). Acetone is added from time to time to keep the volume at 300 ml. or more. After the final addition of the reagents, add 100 ml. of water and raise the temperature to 75° for fifteen minutes in order to distill the bulk of the acetone. The trimethylinulin will appear as pellets or as a fine, porous solid, depending upon the rate of stirring. Filter hot and reflux the crude trimethylinulin with 7 ml. of water for two hours. After three such treatments with boiling water and trituration with acetone and ether, a fine white powder results in a 95% yield. In order to obtain a fully methylated product it is necessary to follow the directions closely;  $[\alpha]_D^{20} = -55^\circ$  ( $c = 1.03$ , chloroform),  $[\alpha]_D^{20} = -54^\circ$  ( $c = 1.09$ , benzene), m. p. 140° (softening to a colorless liquid).

Haworth and Learner<sup>27</sup> determined the mode of linkage by which the D-fructose residues are joined in inulin by hydrolyzing trimethylinulin and identifying the 3,4,6-trimethyl-D-fructofuranose thus formed. Their proof of the structure of this trimethyl-D-fructose follows. Upon oxidation of the trimethyl-D-fructose (I) with nitric acid, a monobasic acid, trimethyl-D-fructofuronic acid (II), resulted. On methylation this gave a tetramethyl methyl ester which formed a crystalline amide. Upon further oxidation with acid permanganate, the trimethyl acid formed crystalline 2,3,5-trimethyl-D-arabonolactone (III). This compound,<sup>27a</sup> when degraded with nitric acid, gave a dimethyltartaric acid (IV) which

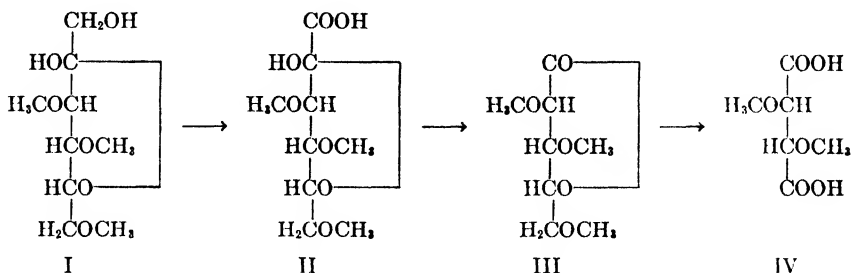
(25) K. Rast, *Ber.*, **55B**, 1051, 3727 (1922).

(26) H. Pringsheim and A. Aronowsky, *Ber.*, **54**, 1281 (1921).

(27) W. N. Haworth and A. Learner, *J. Chem. Soc.*, 619 (1928).

(27a) W. N. Haworth, E. L. Hirst and A. Learner, *J. Chem. Soc.*, 2432 (1927).

was shown to be identical with the dimethyltartaric acid prepared from D(*levo*)-tartaric acid by formation of its crystalline diamide from methylamine.



Inulin is therefore made up of D-fructofuranose residues joined through carbons 1 and 2. The properties of inulin point to a symmetrical arrangement and hence carbon 2 of each D-fructose residue is joined to carbon 1 of an adjacent one.

## 2. Levans

The literature contains many references to the synthesis of levulosans by bacterial action. These substances are known as levans and so far they have all been found to be polysaccharides consisting of fructofuranose residues in which the second or reducing carbon of one is joined to the sixth carbon of an adjacent residue. The levans have been discussed in a preceding chapter (page 225).

## 3. Other Polyfructosans

A large number of polyfructosans that have been reported from time to time by different authors have been investigated by Schlubach and his associates. In order to obtain polysaccharides of constant optical rotation, 100 to 300 precipitations from aqueous solution by the addition of alcohol were necessary. Fifty to 150 precipitations from chloroform solution with petroleum ether were required for purification of the acetate derivatives. These were methylated according to the procedure of Haworth and Streight,<sup>24</sup> and upon hydrolysis partially methylated fructoses were obtained.

It is believed by Schlubach and Sinh<sup>25</sup> that the polyfructosans are, as a rule, made up of D-fructose residues joined in a ring. According to these authors, when the methyl derivatives of the polysaccharides are hydrolyzed, the amounts of dimethyl- and tetramethyl-D-fructose formed

(35) H. H. Schlubach and O. K. Sinh, *Ann.*, **544**, 111 (1940).

indicate the presence of branched chains in the polysaccharide structure. Haworth's end-group method assumes that the tetramethyl-D-fructose formed in relatively small amounts along with trimethyl-D-fructose represents a terminal D-fructose residue in a straight-chain compound. The determination of the relative amounts of each partially methylated fructose requires careful manipulation and study because many of the products are not known in crystalline form.

Schlubach and Sinh<sup>35</sup> have placed the polyfructosans in the following groups, which depend upon the partially methylated fructose formed by hydrolysis of the methylated polysaccharide: inulin, asparagosin, sinistrin, and graminin yield 3,4,6-trimethyl-D-fructofuranose; phlein, levan, poan (poain), and secalin yield 1,3,4-trimethyl-D-fructofuranose; methylated irisin, upon hydrolysis forms only dimethyl-D-fructose and tetramethyl-D-fructose; triticin appears to yield a trimethylfructose that differs from the known trimethyl-D-fructoses; and the polyfructosan designated asphodelin is not known to be a homogeneous product. Brief descriptions of this work follow.

*a. Asparagosin.* Asparagosin was first isolated by Tanret<sup>36</sup> from asparagus roots and later by Schlubach and B  e<sup>37</sup> who used the following procedure. The fresh roots were extracted with water and defecated with lead acetate. The clarified solution was systematically fractionated by the addition of alcohol. There were required 180 such precipitations before a product having a constant rotation was obtained;  $[\alpha]_D^{20} = +32.6^\circ$  ( $c = 1$ , water). Cryoscopic molecular weight determinations in water solution gave an average molecular weight of 1635 for the original polysaccharide and 1505 for that formed by deacetylation of the acetyl derivative.

The polysaccharide was acetylated with acetic anhydride in the presence of pyridine. Purification of the acetate consisted in precipitation of a 10% boiling benzene solution with petroleum ether. One hundred and fifty such precipitations were necessary before a constant-rotating product resulted;  $[\alpha]_D^{20} = -20.1^\circ$  ( $c = 1.0$ , chloroform). Cryoscopic molecular weight determinations in benzene solution gave an average value of 3918.

The acetate was dissolved in acetone and converted to the methyl derivative by the use of dimethyl sulfate and sodium hydroxide. After three methylations, a product containing 45.6%  $\text{OCH}_3$  was obtained. Hydrolysis yielded dimethyl-, trimethyl-, and tetramethyl-D-fructose in the ratios (calculated as D-fructose) of 1:8:1. The trimethyl-D-fructose

(36) G. Tanret, *Bull. soc. chim.*, [4] 5, 889 (1909).

(37) H. H. Schlubach and H. B  e, *Ann.*, 532, 191 (1937).

was identified as 3,4,6-trimethyl-D-fructofuranose by preparation of its crystalline phenylosazone; m. p., 78–79°.

b. *Sinistrin*.<sup>38–40</sup> Many investigators have studied the polyfructosans obtained from the sea onion (*Scilla maritima*, *Urginea maritima*). Of these, sinistrin as described by Schlubach and Loop<sup>40</sup> will be considered here. Sinistrin was purified by alcohol precipitation of an aqueous solution. Two hundred such precipitations were required before a constant-rotating product was obtained. The rotation was made on a sample in which the alcohol was replaced by water and finally dried for two hours at 40° and 12 mm. pressure, followed by three hours of drying at 80° and 0.01 mm. pressure;  $[\alpha]_D^{20} = -45.5^\circ$  ( $c = 1$ , water).

The acetyl derivative was prepared according to the procedure of Haworth and Streight.<sup>24</sup> After 110 precipitations of a warm benzene solution with petroleum ether, a product rotating  $-23.5^\circ$  resulted. Cryoscopic molecular weight determinations in benzene solution gave an average value of 9517, corresponding to 30 to 31 D-fructose residues.

Methylation was accomplished by direct conversion of the acetyl derivative as previously described. The final product contained 45%  $\text{OCH}_3$ . Molecular weight determinations in benzene gave values representing 18 to 19 D-fructose residues. Hydrolytic products of the methyl derivative were separated by conversion to benzoyl derivatives. The trimethylfructose was identified as 3,4,6-trimethyl-D-fructofuranose by its phenylosazone. The authors conclude that the ratios 1:3:1 exist among the di-, tri-, and tetramethyl-D-fructoses produced by the hydrolysis.

c. *Graminin*.<sup>41–55</sup> Graminin was separated by Schlubach and Koenig<sup>55</sup> from rye. Approximately 100 precipitations with alcohol from aqueous

(38) H. H. Schlubach and W. Flörsheim, *Ber.*, **62**, 1491 (1929).

(39) H. Colin and A. Chaudun, *Bull. soc. chim. biol.*, **15**, 1520 (1933).

(40) H. H. Schlubach and W. Loop, *Ann.*, **523**, 130 (1936).

(41) O. Popp, *Ann.*, **156**, 181 (1870).

(42) A. Muntz, *Compt. rend.*, **87**, 679 (1878).

(43) A. G. Eckstrand and C. J. Johanson, *Ber.*, **20**, 3310 (1887).

(44) A. G. Eckstrand and C. J. Johanson, *Ber.*, **21**, 594 (1888).

(45) C. Tanret, *Compt. rend.*, **112**, 293 (1891).

(46) C. Tanret, *Bull. soc. chim.*, [3] **5**, 724 (1891).

(47) E. Schulze and S. Frankfurt, *Ber.*, **27**, 65, 3525 (1894).

(48) E. Jessen-Hansen, Carlsberg-Laboratories, Middelsen, **4**, 145 (1896).

(49) E. Schulze, *Z. physiol. Chem.*, **27**, 267 (1899).

(50) H. Colin and H. Belval, *Compt. rend.*, **175**, 1441 (1922).

(51) H. Colin and H. Belval, *Compt. rend.*, **177**, 343 (1923).

(52) H. Colin, *Bull. soc. chim.*, [4] **8**, 621 (1927).

(53) J. Tillmans, H. Boll and L. Jariwala, *Z. Untersuch. Lebensm.*, **56**, 26 (1928).

(54) C. I. Kruisheer, *Rec. trav. chim.*, **50**, 153 (1930).

(55) H. H. Schlubach and K. Koenig, *Ann.*, **514**, 182 (1934).

solution were required before a constant-rotating product was prepared. Graminin is a white, tasteless, odorless substance having  $[\alpha]_{\text{D}}^{20} = -36.6^\circ$  (water). The average molecular weight determined cryoscopically in water solution was 976. The average molecular weight of the deacetylated product was 494.

Acetylation with acetic anhydride in pyridine solution yielded a product which, after 50 precipitations with petroleum ether from warm benzene solution, gave  $[\alpha]_{\text{D}}^{20} = -7.2^\circ$  ( $c = 1.0$ , chloroform) and an average molecular weight of 2700 (cryoscopically in benzene solution).

Methylgraminin was obtained as a colorless sirup by the method of Haworth and Streight.<sup>24</sup> Hydrolysis of the methyl derivative gave a mixture of di-, tri-, and tetramethyl-D-fructoses that were separated after conversion to benzoyl derivatives. The authors conclude that these partially methylated D-fructoses are present in the ratios 1:1:1 or 1:2:1.

*d. Phlein.* Phlein was isolated first by Eckstrand and Johanson<sup>43,44</sup> and later studied by Schlubach and Sinh.<sup>56</sup> Like levan, its methyl derivative, upon hydrolysis, yields 1,3,4-trimethyl-D-fructose. The latter authors used tubers of *Phleum pratense*, nodosum L variety (timothy), as a source. The roots were ground in the presence of calcium carbonate and the resulting pulp was extracted with water at  $40^\circ$  for one hour and pressed. After defecation with lead acetate, the sirup was diluted with water and 95% alcohol added. The crude phlein separated as a colored precipitate in about 3.2% yield (based on the weight of the roots). After six separations by dissolving in water at  $80^\circ$ , cooling to room temperature, and adding alcohol, a product having a constant rotation was obtained. Since the polysaccharide is unstable at high temperatures, the dry sample was obtained by heating at  $40^\circ$  and 15 mm. pressure, followed by heating at  $60^\circ$  and 0.001 mm. pressure;  $[\alpha]_{\text{D}} = -50.0^\circ$  ( $c = 1.0$ , water).

Phlein was acetylated by acetic anhydride in pyridine solution. The crude product was purified by dissolving the dry substance in as small an amount of chloroform as possible, centrifuging to separate impurities, and precipitating the acetate by the dropwise addition of alcohol. After the process had been repeated a few times, a constant-rotating product resulted.

Methylation was carried out according to the procedure of Haworth and Streight.<sup>24</sup> Trimethylphlein has m. p.  $172^\circ$ ,  $[\alpha]_{\text{D}}^{20} = -57.7^\circ$  ( $c = 1.0$ , chloroform), average molecular weight 3280. Upon hydrolysis, 1,3,4-trimethyl-D-fructose resulted accompanied by a small amount of dimethyl-D-fructose which could be accounted for by the slightly low

methoxyl content of the trimethylphlein used. The authors conclude that phlein is a ring compound consisting of 15 to 16 D-fructose residues in which the second or reducing carbon of each residue is attached to the sixth carbon of an adjacent D-fructose residue.

*e. Poan.* Poan, isolated from *Poa trivialis* (roughstalk bluegrass) by Challinor, Haworth and Hirst,<sup>57</sup> has properties very similar to those of levan. Hydrolysis of the methyl derivative yields 1,3,4-trimethyl-D-fructose as the main product. Since a quantitative analysis was not made, these authors were unable to state whether levan and poan are identical, differing only in degree of purification, or whether they are distinct compounds.

*f. Secalin.* Secalin has been isolated from the stems of unripe rye.<sup>45, 58</sup> Schlubach and Bandmann<sup>59</sup> studied its structure. The great difficulty they encountered in obtaining the polysaccharide and its acetate in homogeneous form made the determination of physical properties uncertain. However, by hydrolysis of the methyl derivative, they obtained, after separation by means of the  $\beta$ -naphthoates, tetramethyl-, trimethyl-, and dimethyl-D-fructoses in the proportions of 1:2:1. The trimethyl-D-fructose was identified as 1,3,4-trimethyl-D-fructofuranose by its melting point and specific rotation.

*g. Triticin.* Triticin is a polyfructosan occurring in *Agropyron repens* (quackgrass, couchgrass). Although previous investigators<sup>52, 60, 61</sup> isolated a polysaccharide from the same source, the product described here was isolated by Schlubach and Peitzner.<sup>62</sup>

Three hundred precipitations of the aqueous solution with alcohol were necessary before a constant-rotating product was obtained.  $[\alpha]_{\text{D}}^{20} = -51.4^\circ$  (water). (Alcohol was removed from the sample by repeated evaporation with water followed by drying for two hours at  $80^\circ$  in a high vacuum.) Cryoscopic determination of the molecular weight in water solution gave an average of 2600 for the original and 2825 for the deacetylated product.

Triticin was acetylated according to the procedure of Haworth and Streight.<sup>24</sup> After 490 precipitations from chloroform solution by addition of petroleum ether, a product having a constant rotation resulted;  $[\alpha]_{\text{D}}^{20} = -15.5^\circ$  ( $c = 2$ , chloroform) (dried for two hours in a high vacuum at  $60^\circ$ ). This acetate separated in two forms, one soluble in

(57) S. W. Challinor, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, 1560 (1934).

(58) H. Belval, *Rev. Gen. Botany*, **36**, 308 (1924).

(59) H. H. Schlubach and C. Bandmann, *Ann.*, **540**, 285 (1939).

(60) C. Tanret, *Bull. soc. chim.*, [3] **5**, 724 (1891).

(61) H. Colin and A. de Cugnac, *Bull. soc. chim. biol.*, **8**, 621 (1926).

(62) H. H. Schlubach and H. Peitzner, *Ann.*, **530**, 120 (1937).

benzene (m. p. 115°) and the other soluble in acetone (m. p. 191°). Molecular weight determinations in benzene solution gave very irregular results.

*h. Irisin.* Irisin was first prepared by Wallach<sup>63</sup> from the roots of *Iris pseudacorus*. He obtained it as a white, hygroscopic compound having  $[\alpha]_{\text{D}}^{20} = -51.5^\circ$  ( $c = 10$ , water). It formed D-fructose on acid hydrolysis and did not reduce Fehling solution. Wallach believed it to be a polysaccharide different from inulin because of its higher rotation, its different appearance when deposited from an aqueous solution, and its greater solubility in cold water.

Euler and Erdtmann<sup>64</sup> obtained irisin from the same source and reported  $[\alpha]_{\text{D}}^{20} = -52.6^\circ$  (water), m. p. 200–204° for the product regenerated from the acetate. By treatment of the acetate with phenylhydrazine they reported a nonreducing product having  $[\alpha]_{\text{D}}^{20} = -49$  to  $-50^\circ$  (water). This gave a molecular weight by the diffusion method amounting to about one-third that of the original irisin. When acetylated, the product was more readily soluble in methyl alcohol than the original acetylated irisin.

They prepared the acetate by means of acetic anhydride in dry pyridine and purified it by separation from an acetic acid solution to which warm methyl alcohol had been added;  $[\alpha]_{\text{D}}^{20} = -22.70^\circ$  ( $c = 10$ , acetic acid), m. p. 206–208°. In the presence of barium hydroxide irisin formed an insoluble product whose composition was represented by  $6(\text{C}_6\text{H}_{10}\text{O}_5) \cdot \text{Ba}(\text{OH})_2$ . In contrast to inulin, these authors found that Takadiastase at pH 5 to 5.5 did not hydrolyze irisin.

Schlubach, Knoop and Liu<sup>65</sup> investigated the structure of irisin by hydrolysis of the methylated polysaccharide. The acetyl derivative was converted to methylirisin by one treatment with dimethyl sulfate and sodium hydroxide in acetone solution. It was obtained as a white powder having  $[\alpha]_{\text{D}}^{20} = -63.2^\circ$  ( $c = 2.14$ , chloroform), m. p. 188–190°.

Upon hydrolysis an equal amount of tetramethyl-D-fructose and dimethyl-D-fructose was obtained. The first of these was identified as 1,3,4,6-tetramethyl-D-fructose by conversion to the characteristic crystalline acid amide obtained by Haworth, Hirst and Nicholson.<sup>66</sup> The dimethyl-D-fructose was obtained as a thick brown sirup having  $[\alpha]_{\text{D}}^{20} = +20.0^\circ$  ( $c = 1.82$ , chloroform).

(63) O. Wallach, *Ann.*, **234**, 364 (1886).

(64) H. v. Euler and H. Erdtmann, *Z. physiol. Chem.*, **145**, 261 (1925).

(65) H. H. Schlubach, H. Knoop and M. Y. Liu, *Ann.*, **504**, 30 (1933).

(66) W. N. Haworth, E. L. Hirst and V. S. Nicholson, *J. Chem. Soc.*, **129**, 1518 (1927).

From these results the authors conclude that irisin is composed of a series of D-fructose units, each member having an additional D-fructose molecule attached. The tetramethyl-D-fructose is thus derived from these dangling D-fructose units. The position of the unions in the rest of the molecule has not been ascertained since the structure of the dimethyl-D-fructose is unknown.

The same authors further studied the acid hydrolysis of the original irisin and also of the product regenerated from the acetyl derivative. They believe that the hydrolysis of irisin can be represented by the following scheme.

Irisin  $\rightarrow$  tetrafructose anhydride  $\rightarrow$  difructose anhydride  $\rightarrow$  D-fructose. The difructose anhydride, although not isolated in pure form, was readily hydrolyzed by acid and thus differed from the difructose anhydrides isolated from inulin.

## II. DIFRUCTOSE ANHYDRIDES

Four difructose anhydrides have been isolated as well-defined crystalline compounds. The properties of these substances and their derivatives are recorded in Table II.

### 1. Diheterolevulosan

Diheterolevulosan was first prepared by Pictet and Chavan<sup>67</sup> by treating D-fructose with concentrated hydrochloric acid. Schlubach and Behre<sup>68</sup> prepared the same compound by the action of liquid hydrogen chloride on dry fructose in a sealed tube. Sattler and Zerban<sup>69</sup> have found this difructose anhydride in the unfermentable residue obtained from cane molasses.

Schlubach and Behre<sup>68</sup> found the hexamethyldiheterolevulosan difficult to hydrolyze; however, they were able to obtain a trimethyl-D-fructose,  $n_D^{20} = 1.4723$ ;  $[\alpha]_D^{20} = -55.2^\circ$  ( $c = 1.07$ , chloroform);  $[\alpha]_D^{20} = -73.5^\circ$  ( $c = 0.70$ , water). The phenylosazone of this trimethyl-D-fructose did not crystallize, but was isolated as a heavy oil. Analysis showed that it contained three methoxyl groups. The authors thus concluded that carbons 1 and 2 must not contain methoxyl groups. Because of its high negative rotation, they assumed that it contained a pyranose ring, and consequently was 3,4,5-trimethyl-D-fructopyranose. They thus concluded that diheterolevulosan is a di-D-fructopyranose

(67) A. Pictet and J. Chavan, *Helv. Chim. Acta*, **9**, 809 (1926).

(68) H. H. Schlubach and C. Behre, *Ann.*, **508**, 16 (1933).

(69) L. Sattler and F. W. Zerban, *Sugar*, **39**, 28 (1944); *Ind. Eng. Chem.*, **37**, 1133 (1945).



TABLE II  
*Di-D-fructose Anhydrides and Derivatives*

	Melting point	Boiling point	$n_D^{20}$	$[\alpha]_D^{20}$	Concentration	Solvent	Reference
Diheterolevulosan	°C. 266-267	°C. —	—	—43.34 <sup>a</sup>	g./100 ml. 4.1	Water	68
Hexaacetyl-diheterolevulosan	171-173	—	—	-41.7 -59.1	1.01 1.02	Benzene Chloroform	68
Hexamethyl-diheterolevulosan	143-145	—	—	-46.5	1.00	Chloroform	68
Di-D-fructose anhydride I	164	—	—	+26.9	8.45	Water	70, 71, 72
Hexaacetyl-di-D-fructose anhydride I	137, sinters at 125	—	—	+ 0.54	9.98	Chloroform	70, 72
Hexamethyl-di-D-fructose anhydride I	—	170-175, 0.01 mm.	—	+19.1 +23.7 +50.4	— 4.86 1.61	None Chloroform Water	73, 75
6,6'-Ditrityl-di-D-fructose anhydride I	195	—	—	+20.3	2.18	Chloroform	75

<sup>a</sup> Measured at 18°C.

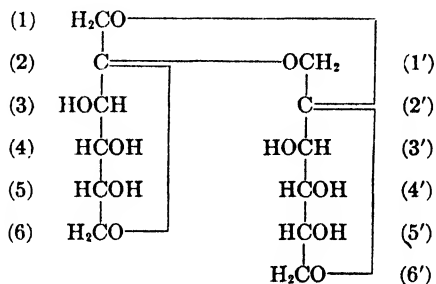
TABLE II (Continued)

	Melting point	Boiling point	$n_D^{20}$	$[\alpha]_D^{20}$	Concentration	Solvent	Reference
6,6'-Ditriyl-3,4:3',4'-tetraacetyl-di-D-fructose anhydride I	°C. 194	°C. —	—	+21.06	g./100 ml. 3.73	Chloroform	75
3,4:3',4'-Tetraacetyl-di-D-fructose anhydride I	173	—	—	-9.9	3.73	Chloroform	75
6,6'-Dimethyl-3,4:3',4'-tetraacetyl-di-D-fructose anhydride I	127-128	—	—	+10.8	1.23	Chloroform	75
Di-D-fructose anhydride II	198	—	—	+13.85	8.99	Water	74
Hexamethyl-di-D-fructose anhydride II	73	169-170, 0.35 mm.	1.4673 <sup>b</sup>	-41.9 <sup>b</sup> -28.2 +6.0	— 3.55 3.73	None Chloroform Water	75
Di-D-fructose anhydride III	162	—	—	+135.64	7.84	Water	74, 75
Hexamethyl-di-D-fructose anhydride III	—	161-165, 0.42 mm.	1.4658	+155.7 +157.9 +164.5	— 3.95 2.23	None Chloroform Water	75
6,1',6'-Tri(trityl)-di-D-fructose anhydride III	127	—	—	+64.2	0.76	Chloroform	75

<sup>b</sup> Undercooled.

anhydride in which the reducing carbon of each D-fructose unit is joined to the first carbon of the other D-fructose unit.

The evidence from the work of Schlubach and Behre<sup>68</sup> is taken to indicate that diheterolevulosan possesses the structural formula that is here shown (X).



X

Diheterolevulosan  
(1,2':2,1'-Di-D-fructopyranose anhydride)

Molecular weight measurements agreed with the difructose anhydride composition. The matter of the anomeric structures of the D-fructose units is left undecided because there is no evidence now known which relates to this question.

## 2. Difructose Anhydride I

Difructose anhydrides I, II, and III have been isolated from the nonreducing residue that remains after removal of D-fructose and D-glucose from acid-hydrolyzed inulin.

Difructose anhydride I was first prepared by Jackson and Goergen.<sup>16,70</sup> These authors found a molecular weight of 307 for the anhydride and 574 for the acetate, values which indicate its difructose anhydride composition. Almost simultaneously, Irvine and Stevenson<sup>71</sup> prepared the acetate of what they believed to be an anhydrofructose by the action of concentrated nitric acid on inulin acetate. This anhydride was later shown<sup>72</sup> to be identical with the difructose anhydride I of Jackson and Goergen. Hexamethyldifructose anhydride I was hydrolyzed by Haworth and Streight<sup>73</sup> and the trimethyl-D-fructose that was formed was identified as 3,4,6-trimethyl-D-fructofuranose by quantitative conversion

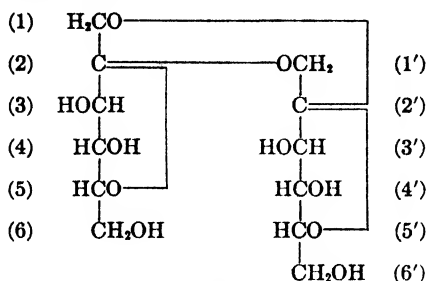
(70) R. F. Jackson and Sylvia M. Goergen, *Bur. Standards J. Research*, **5**, 733 (1930).

(71) J. C. Irvine and J. W. Stevenson, *J. Am. Chem. Soc.*, **51**, 2197 (1929).

(72) E. W. Bodycote, W. N. Haworth and C. S. Woolvine, *J. Chem. Soc.*, 2389 (1932).

(73) W. N. Haworth and H. R. L. Streight, *Helv. Chim. Acta*, **15**, 693 (1932).

into its phenylosazone. Difructose anhydride I is therefore a 1,2':2,1'-di-D-fructofuranose anhydride, the structural formula of which is here shown by XI, without specification concerning  $\alpha$  or  $\beta$  linkages, since there is no evidence on that subject.



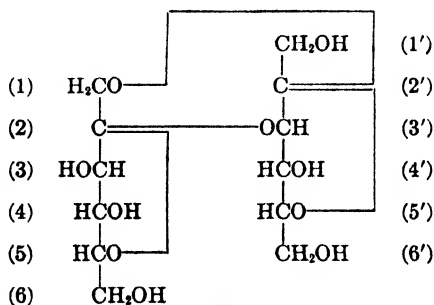
XI

Difructose anhydride I  
(1,2':2,1'-Di-D-fructofuranose anhydride)

### 3. Difructose Anhydride III

Difructose anhydrides II and III were first reported by Jackson and McDonald.<sup>74</sup> In both cases molecular weight determinations indicated a difructose anhydride molecule. Difructose anhydride III has been shown by the following procedure<sup>75</sup> to be 1,2':2,3'-di-D-fructofuranose anhydride.

The structural formula, without specification of  $\alpha$  or  $\beta$  linkages, since these are unknown, is shown by XII.



XII

Difructose anhydride III  
(1,2':2,3'-Di-D-fructofuranose anhydride)

(74) R. F. Jackson and Emma J. McDonald, *Bur. Standards J. Research*, **6**, 709 (1931).

(75) Emma J. McDonald and R. F. Jackson, *J. Research Natl. Bur. Standards*, **24**, 181 (1940).

The proof for the formula XII is as follows. The full methylation of difructose anhydride III yielded a hexamethyl derivative, non-crystalline but readily distillable at 161–165° in a vacuum of 0.42 mm. The hydrolysis of hexamethyl-difructose anhydride III in 0.8 *N* aqueous hydrochloric acid at 95° appeared to reach half completion in about ninety-one minutes; it is evident that the substance is rather difficult to hydrolyze. There is some destruction of the hydrolytic products in the acid solution with the formation of furfural derivatives, and it was found best to stop the hydrolysis at the half stage and separate the trimethyl-D-fructose fraction from the unhydrolyzed material by distillation in vacuo, the hydrolyzed products being more volatile; the still residue was again hydrolyzed to the half stage and the separation repeated. In this manner there was obtained a yield of about 58% redistilled trimethyl-D-fructose, the specific rotation of which in chloroform\* is in the range  $[\alpha]_D = +25-30^\circ$ . Although this value is near that of pure 3,4,6-trimethyl-D-fructose, which is  $+26.6^\circ$ , the agreement proved to be fortuitous because the sirup was not solely 3,4,6-trimethyl-D-fructose. It contained 3,4,6-trimethyl-D-fructose (readily identified by oxidation to 2,3,5-trimethyl-D-arabonolactone), but in addition a second trimethyl-D-fructose, the identification of which as 1,4,6-trimethyl-D-fructose was accomplished through a series of reactions that will be described in the continuation. The structural formula XII for difructose anhydride III is deduced from the structures of the two foregoing trimethyl-D-fructoses.

A portion of the mixture of trimethyl-D-fructoses was converted to their methyl D-fructosides, which were then completely methylated. After removal of the D-fructosidic methyl groups, the resulting tetramethyl D-fructose had a specific rotation  $[\alpha]_D = +37^\circ$  (water), showing that both the trimethyl-D-fructoses in the distillate belonged to the furanose series and passed to the single 1,3,4,6-tetramethyl-D-fructose in the later operations. The rotation of  $+37^\circ$  clearly distinguishes this tetramethyl-D-fructofuranose from 1,3,4,5-tetramethyl-D-fructose, the D-fructopyranose form, which is strongly levorotatory. With the hemiacetal ring in the 2,5-position (fructofuranose type), there are four possible trimethyl-D-fructoses, namely 1,3,4; 1,3,6; 1,4,6; and 3,4,6. The distillate contained as one constituent 3,4,6-trimethyl-D-fructose, together with one of the remaining three. From these, 1,3,4 can be eliminated. Hibbert, Tipson and Brauns<sup>75a</sup> have prepared this substance

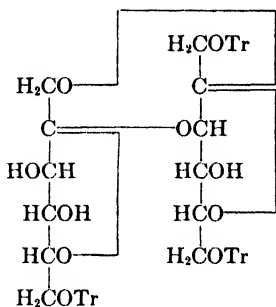
\* The rotations were measured in chloroform solution, a fact which is not stated in the original article. The hydrolysis curves for aqueous solution of difructose anhydride III indicate that the rotation in water of the resulting trimethyl-D-fructose is also within this range.

(75a) H. Hibbert, R. S. Tipson and F. Brauns, *Can. J. Research*, **4**, 221 (1931).

by the hydrolysis of fully methylated levan (see page 227); the pure 1,3,4-trimethyl-D-fructose is crystalline and shows  $[\alpha]_D = -51.4^\circ$  (water). If this trimethyl-D-fructose had been produced together with the 3,4,6-form of  $[\alpha]_D = +30.5^\circ$  (water) by the hydrolysis of hexamethyl-difructose anhydride III, the resulting sirupy mixture of trimethyl-D-fructoses would have been levorotatory, whereas its rotation was in the range  $+25$ – $30^\circ$ . Therefore, the products of hydrolysis were 3,4,6- and either 1,3,6- or 1,4,6-trimethyl-D-fructose. The proof that the second component is really the 1,4,6-trimethyl-D-fructose will now be described.

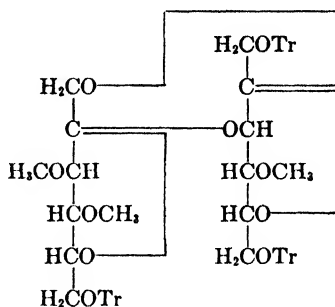
Difructose anhydride III yields by reaction in pyridine solution with triphenylchloromethane (trityl chloride) a pure crystalline tritryl derivative (XIII) (page 272), which in turn yields an amorphous tri(trityl)-triacetyl derivative on acetylation. The methylation of the last substance in aqueous acetone by dimethyl sulfate and sodium hydroxide gives an amorphous tri(trityl)-trimethyl-difructose anhydride III (XIV) (page 272). This substance was detritylated in chloroform solution containing hydrobromic acid and the resulting trimethyl-difructose anhydride III, together with the hydrobromic acid, was extracted with water and hydrolyzed to form a mixture of partially methylated D-fructoses, which were then converted to the methyl D-fructosides and the mixture distilled. The lower-boiling fraction consisted of methyl dimethyl-D-fructoside; its hydrolysis yielded a dimethyl-D-fructose, the phenylosazone of which proved to be identical with that of 3,4-dimethyl-D-fructose. [Authentic 3,4-dimethyl-D-fructose, hitherto unknown, was prepared from difructose anhydride I (formula XI on page 269) through the methylation in alkaline aqueous acetone solution of the pure crystalline 6,6'-ditrityl-3,4:3',4'-tetraacetyl-di-D-fructose anhydride I, followed by detritylation and hydrolysis of the amorphous 3,4:3',4'-tetramethyl-di-D-fructose anhydride I.] The lower-boiling fraction was thus identified as methyl 3,4-dimethyl-D-fructoside. The higher-boiling fraction proved to be a methyl monomethyl-D-fructoside, which yielded by hydrolysis a monomethyl-D-fructose, the phenylosazone of which proved to be identical with the known phenylosazone of 4-methyl-D-glucose. The higher-boiling fraction was therefore methyl 4-methyl-D-fructoside. In summary, the methylation of the tritylated difructose anhydride III (XIII) led eventually to two partially methylated D-fructoses which were identified as 3,4-dimethyl-D-fructose and 4-monomethyl-D-fructose. The 3,4-dimethyl-D-fructose was evidently derived from the same D-fructose unit that had yielded 3,4,6-trimethyl-D-fructose by hydrolysis of the hexamethyl-difructose anhydride III. The 4-monomethyl-D-fructose was evidently derived from the other D-fructose unit which had

yielded the trimethyl-D-fructose that has already been limited to the 1,3,6 or 1,4,6 structure. The latter of these two possibilities must now be selected, since the identification of 4-methyl-D-fructose is compatible only with it. The final result, therefore, shows that difructose anhydride III possesses the structure XII (page 269).



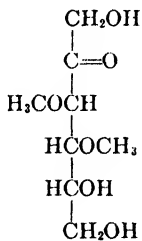
XIII

Tri(trityl)-di-D-fructose  
anhydride III



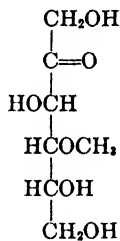
XIV

Tri(trityl)-trimethyl-di-D-fructose  
anhydride III



XV

3,4-Dimethyl-D-  
fructose



XVI

4-Monomethyl-D-  
fructose

It has been mentioned that the mixture of trimethyl-D-fructoses from the acid hydrolysis of hexamethyl-di-D-fructose anhydride III, which are now known to be the 3,4,6- and 1,4,6-trimethyl-D-fructoses, shows a specific rotation near that of pure 3,4,6-trimethyl-D-fructose; it is to be inferred accordingly that these two trimethyl-D-fructoses do not differ greatly in rotation. Montgomery<sup>76</sup> has synthesized 1,4,6-trimethyl-D-fructose and found its rotation in chloroform to be  $[\alpha]_D = +29.7^\circ$ , a value approximating that of 3,4,6-trimethyl-D-fructose ( $+27.7^\circ$  in the same solvent). Pertinent also are the respective rotations of the acetone condensation products from these two trimethyl-D-fructoses. Montgomery found that 3,4,6-trimethyl-D-fructose under-

(76) T. N. Montgomery, *J. Am. Chem. Soc.*, **56**, 419 (1934).

went a large increase in dextrorotation upon condensation with acetone, an observation which McDonald and Jackson<sup>75</sup> verified, while 1,4,6-trimethyl-D-fructose changed but slightly. The rotation of the mixed trimethyl-D-fructoses from the hydrolysis of hexamethyl-di-D-fructose anhydride III, upon condensation with acetone, increased by an amount roughly intermediate between that found for 3,4,6- and that shown by Montgomery's 1,4,6-trimethyl-D-fructose.

#### 4. Difructose Anhydride II

The structure of difructose anhydride II has not yet been determined. McDonald and Jackson<sup>75</sup> prepared its hexamethyl ether, which proved to be a crystalline compound melting at 73°. The behavior of the product from the acid hydrolysis of this hexamethyl derivative toward phenylhydrazine suggests that two different trimethyl-D-fructoses are present, the identification of which remains to be accomplished. Lately some evidence concerning the structure of this anhydride has been obtained by the use of per-iodic acid oxidation, as described on page 275.

### III. MISCELLANEOUS

#### 1. Rates of Hydrolysis by Acids

The polyfructosans, in contrast to the difructose anhydrides, are readily hydrolyzed in acid solution. Schlubach and his associates<sup>38</sup> have measured the rates of hydrolysis of a large number of polyfructosans. The time required for 50% hydrolysis to take place in *N* sulfuric acid solution at 20° is recorded in Table III. These results are based on

TABLE III  
*Half Periods for Hydrolysis of Polyfructosans in N Sulfuric Acid at 20° C.*  
Data of Schlubach and Sinh<sup>38</sup>

Polyfructosan	Minutes	Polyfructosan	Minutes
Asparagosin	300	Phlein	235
Asphodelin	288	Secalin	225
Graminin	222	Sinistrin	269
Inulin	360	Triticin	216
Irisin	242		

reducing-sugar determinations. Polariscopic data, when obtained, indicated similar hydrolysis rates except in the case of irisin. Here the authors conclude that intermediate products are formed.



The difructose anhydrides are much more stable toward acid hydrolysis. Using 0.2 *N* acid at 100°, Jackson and Goergen<sup>70</sup> found that difructose anhydride I was 84.3% hydrolyzed in 100 minutes. Under the same conditions difructose anhydrides II and III were hydrolyzed,<sup>75</sup> respectively, 64% in 100 minutes and 81% in 110 minutes. Schlubach and Knoop<sup>77</sup> report the hydrolysis rates of difructose anhydride I and diheterolevulosan shown in Table IV.

TABLE IV  
*Half Periods of Acid Hydrolysis in N Sulfuric Acid*  
Data of Schlubach and Knoop<sup>77</sup>

	At 20°C. (Minutes)	At 60°C. (Minutes)
Difructose anhydride I	220,000	463
Diheterolevulosan	—	1,071
Sucrose	262	—

Haworth and Streight<sup>73</sup> believe that the stability of difructose anhydride I to acid can be attributed to the presence of the dioxane ring. The similar stability of diheterolevulosan and of difructose anhydride III, both of which contain the dioxane group, agrees with this interpretation.

## 2. Origin of Difructose Anhydrides from Inulin

A possible mechanism for the origin of the difructose anhydrides from inulin has been suggested by McDonald and Jackson<sup>75</sup> in the following paragraph:

"We may suppose that during the process of hydrolysis the inulin aggregate is ruptured at various points, leaving shortened chains, each fragment having a free reducing group at one end. In a relatively small number of instances the hydroxyl of the terminal reducing group apparently condenses with one of the hydroxyls of the penultimate D-fructose residue, thus forming a di-D-fructose anhydride entity which becomes so stable as to resist further hydrolysis. This condensation of one D-fructose residue with a closely contiguous residue is in keeping with the known tendency of fructose derivatives to polymerize. On the penultimate D-fructose residue, carbon atoms 3, 4, and 6 bear hydroxyls which are available for this condensation, and the union through an atom of oxygen of carbon atom 3 with carbon 2 of the terminal residue would

(77) H. H. Schlubach and H. Knoop, *Ann.*, **504**, 19 (1933).

lead to the formation of difructose anhydride III. This condensation clearly can occur at any time before the complete resolution of the inulin fragments to individual D-fructose units. Di-D-fructose anhydride I, on the other hand, can only be formed by the momentary isolation of a fragment consisting of two D-fructose residues, followed by condensation or, obviously, by the simultaneous hydrolytic splitting of the fragment and condensation to the anhydride. This latter case is evidently what occurs during the course of Irvine and Stevenson's reaction of inulin acetate in chloroform solution with nitric acid."

Schlubach, Knoop and Liu<sup>65</sup> found indications that a more readily hydrolyzed di-D-fructose anhydride was an intermediate product in the hydrolysis of irisin.

Jackson and Goergen<sup>16</sup> found that when D-fructose was treated with sulfuric acid under conditions similar to those used in preparing the difructose anhydrides from inulin, no condensation of the D-fructose took place.

It is interesting to note that the formation of the dioxane ring stabilizes the anhydrides as to their pyranose or furanose structures, difructose anhydride I as 2,1':1,2'-di-D-fructofuranose and diheterolevulosan as 2,1':1,2'-di-D-fructopyranose.

### 3. Oxidation of Difructose Anhydrides by Per-iodic Acid

Per-iodic acid oxidation<sup>78-80</sup> has been used by many investigators in structural studies because this reagent oxidizes selectively the hydroxyls on adjacent carbon atoms. In the course of their studies, McDonald and Jackson<sup>81</sup> found that difructose anhydrides I, II and III and diheterolevulosan reduce per-iodic acid to iodic acid in the following respective molecular ratios: 2, 1, 1, and 4 moles of acid per mole of anhydride.

Difructose anhydride I (XI, page 269) contains two pairs of adjacent carbon atoms to which hydroxyl groups are attached (3,4 and 3',4'); thus, it would be expected to react with two moles of per-iodic acid. Difructose anhydride III (XII, page 269) contains only one such pair (3,4) in agreement with its consumption of one mole of acid. Diheterolevulosan (X, page 268) requires four moles of acid, one for each of the following pairs of carbon atoms: 3,4; 4,5; 3',4'; 4',5'.

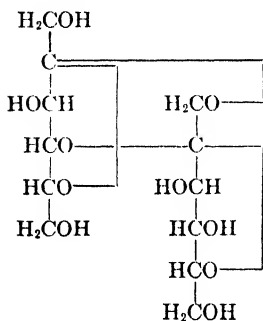
(78) P. Fleury and J. Lange, *J. pharm. chim.*, **17**, 107 (1933).

(79) E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.*, **59**, 994 (1937).

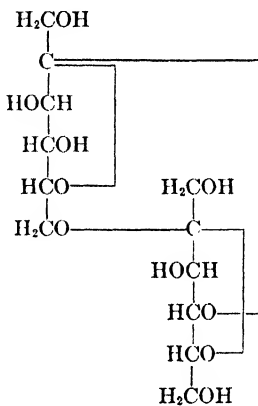
(80) E. L. Jackson, "Organic Reactions," vol. II, p. 341, John Wiley and Sons, Inc., New York (1944).

(81) Emma J. McDonald and R. F. Jackson, *J. Research Natl. Bur. Standards*, **35**, 497 (1945).

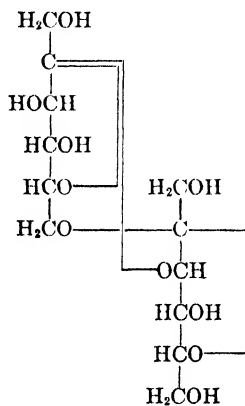
Difructose anhydride II reacts with one mole of per-iodic acid. Hydrolysis of its methyl derivative indicates that two different trimethyl-*D*-fructoses are formed whose combined specific rotations in water amount to between  $+20^\circ$  and  $+30^\circ$ . If we assume that this anhydride, like the other two derived from inulin, is made up of *D*-fructofuranoses, the facts that have been mentioned allow only three possible structures for this anhydride.



XVII



XVIII



XIX

A compound represented by XVIII would yield 1,3,4-trimethyl-*D*-fructose, a crystalline substance with  $[\alpha]_D^{20} = -51.4^\circ$  (water), and 1,3,6-trimethyl-*D*-fructose (now unknown), upon hydrolysis of its hexamethyl derivative. No evidence has been obtained to indicate the presence of this negatively rotating trimethyl-*D*-fructose and it is established that the mixture of trimethyl-*D*-fructoses from hexamethyl-di-*D*-fructose anhydride II rotates in the positive range of 25 to  $30^\circ$ . Unless the 1,3,6-trimethyl-*D*-fructose should prove to possess the unusually high rotation of about  $+100^\circ$ , which seems at least improbable, structure XVIII can be excluded.

If di-*D*-fructose anhydride II has formula XIX, a mixture of 1,3,4- and 1,4,6-trimethyl-*D*-fructoses would be present in the hydrolytic product. Such a mixture would have a specific rotation of  $-10^\circ$  to  $-20^\circ$  (water) in contrast to the value of  $+25$  to  $+30^\circ$  found by McDonald and Jackson.<sup>75</sup> The rotation of 1,4,6-trimethyl-*D*-fructose was measured by Montgomery<sup>76</sup> in chloroform ( $+29.7^\circ$ ), but it has not been measured directly in water. However, the hydrolysis data of McDonald and Jackson<sup>75</sup> for hexamethyl-di-*D*-fructose anhydride III show that 1,4,6-trimethyl-*D*-fructose has about the same rotation in water as in chloroform. The argument thus appears to exclude structure XIX.

An anhydride whose formula is represented by XVII would yield a mixture of 3,4,6-trimethyl-D-fructose and 1,3,6-trimethyl-D-fructose when its hexamethyl derivative was hydrolyzed. Available data concerning di-D-fructose anhydride II are at least in accord with this structure, and if the present inferences are accepted, one may conclude that the rotation of 1,3,6-trimethyl-D-fructose is near those of 1,4,6- and 3,4,6-trimethyl-D-fructose.



# CELLULOSE ETHERS OF INDUSTRIAL SIGNIFICANCE

BY JOSEPH F. HASKINS

*Department of Chemistry, The Ohio State University, Columbus, Ohio*

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## I. PRESENT AND POTENTIAL STATUS OF CELLULOSE ETHERS

Until recent years only three of the many theoretically possible derivatives of cellulose have had any very important commercial development. These are cellulose nitrate,<sup>1</sup> cellulose acetate<sup>1</sup> and cellulose xanthate. All three are esters. The last named is used only as an intermediate in the production of regenerated cellulose, usually in the form of films and filaments. Cellulose nitrate and acetate have found many uses. Each has characteristic advantages, particularly in the field of plastics. Cellulose nitrate is, in most respects, an ideal plastic material because of its compatibility with solvents, plasticizers and softeners. No other plastic material quite equals, in toughness and tensile strength, the combination of cellulose nitrate and camphor which is the oldest of the synthetic plastics. But cellulose nitrate is inflammable and is sensitive to the action of ultraviolet light. Cellulose acetate, on the other hand, is relatively nonflammable and is insensitive to ultraviolet light. It has, however, only a narrow range of solubility and compatibility with plasticizers, which limits its usefulness in the field of plastics. Moreover, being an ester and slightly hygroscopic, it is subject to hydrolysis.

Cellulose ethers of a high degree of substitution are stable, relatively nonflammable, resistant to ultra-violet light and compatible with a wide range of solvents and plasticizers. Certain ethers of a low degree of substitution are soluble in water or alkali. It would appear from these properties that cellulose ethers should be ideally adapted for use in the plastics field and for many other uses.

(1) C. R. Fordyce, *Advances in Carbohydrate Chem.*, **1**, 309 (1945).

While cellulose ethers have been known for many years<sup>1a-4</sup> and while many patents have appeared covering their preparation and uses,<sup>2-4</sup> commercial development has been slow, and only in recent years have these products appeared on the market in any considerable quantities. This slowness in development of the cellulose ethers has come in part from the cost of the alkylating agents but to a much greater extent from the technical difficulties which have to be overcome in the etherification reaction. These difficulties arise from the nature of cellulose and particularly from the colloidal aspects of the reaction by which the ethers are made. As the etherification is usually carried out, cellulose, highly swollen by a concentrated aqueous solution of sodium hydroxide, is treated with an alkylating agent, usually an ester of an inorganic acid. The reaction is a permutoid reaction involving an insoluble but highly hydrophilic colloid (cellulose) reacting, in the presence of water and alkali, with a reagent which is usually relatively insoluble in the alkali but subject to hydrolysis by it. The etherifying agent, in order to reach all of the hydroxyl groups of the cellulose, must diffuse through the alkali and through or between the micells of the cotton or wood pulp which is being used.<sup>5</sup> As a result of this heterogeneity of the reaction mixture, etherification is not uniform and much of the etherifying agent is lost in side reactions.

## II. THE RAW MATERIALS

Cellulose in the form of cotton linters, wood pulp or regenerated cellulose is used as the raw material for the commercial production of cellulose ethers. The advantage which linters possess over wood pulp in the manufacture of cellulose esters is not so apparent in the case of the ethers.

## III. ETHERIFYING AGENTS

The usual etherifying agents are the alkyl chlorides or sulfates. The advantage, usually found in organic reactions, of using the alkyl iodides and bromides because of their greater reactivity, as compared to the chlorides is overshadowed by their much slower diffusion rate, lower solubility in the alkali and greater rate of saponification. The sulfates are relatively costly. Alkyl sulfites have also been proposed<sup>6</sup>

(1a) W. Suida, *Monatsh.*, 26, 413 (1905).

(2) O. Leuchs (to Bayer and Co.), German Pat. 322,586 (1920).

(3) H. Dreyfus, French Pat. 462,274 (1914).

(4) L. Lilienfeld, Brit. Pat. 12,854 (1913); U. S. Pat. 1,188,376 (1916).

(5) E. J. Lorand and E. A. Georgi, *J. Am. Chem. Soc.*, 59, 1166 (1937).

(6) W. Voss and E. Blanke, *Ann.*, 485, 250 (1931).



as etherifying agents. [Sodium methyl (or ethyl) sulfate may be used as the etherifying agent in the preparation of ethers with a low and uniform degree of substitution.] These reagents are soluble in alkali and for this reason they can be distributed uniformly throughout the cellulose. Sodium chloroacetate, which is used in preparing carboxymethyl derivatives,<sup>8</sup> is also soluble in the alkali. [Ethylene oxide is used to prepare the hydroxyethyl ether.<sup>9</sup>] [Because of its high vapor pressure and low molecular weight, ethylene oxide probably distributes itself throughout the cellulose with better uniformity than do the alkyl halides.] [Ethylene chlorohydrin and glycerol monochlorohydrin<sup>10</sup> are effective etherifying agents which probably react through the intermediate formation of ethylene oxide or glycidol.] Epichlorohydrin and dichlorohydrin have been proposed as etherifying agents<sup>11</sup> but it would appear probable that they would produce cross-linking. This statement should be true of other polyfunctional etherifying agents.

The higher alkyl halides have been proposed as etherifying agents but their direct action on alkali cellulose does not give very satisfactory results. Used on a partially ethylated or methylated cellulose, they may be effective.<sup>12,13</sup> Benzyl chloride appears to possess a sufficient solubility and a suitable reactivity to serve as a useful etherifying agent.<sup>5</sup> For patent purposes a great number of halogenated organic compounds have been named as etherifying agents in this reaction<sup>14,15</sup> but it may be questioned whether most of them have any real usefulness.

#### IV. PROCEDURE FOLLOWED IN ETHERIFICATION

Cotton linters or wood pulp, usually in the form of sheets, is steeped in strong alkali (18–50%). The swollen sheets are then pressed to force out most of the excess alkali solution. This "alkali cellulose" is then shredded, and aged if low viscosity is desired. The aging process is the one followed in the viscose industry and is fully explained in any discussion of that process. More alkali may be introduced during the shredding, either as a concentrated solution or as solid alkali.<sup>16</sup> The alkali

(7) R. W. Maxwell, U. S. Pat. 2,101,263 (1937).

(8) E. Jansen, German Pat. 332,203 (1921).

(9) A. W. Schorger and M. J. Shoemaker, *Ind. Eng. Chem.*, **29**, 114 (1937); cf. U. S. Pat. 1,863,208 (1932), 1,914,172 (1933) and 1,941,276–8 (1933).

(10) L. Lilienfeld, U. S. Pat. 1,722,927 (1929).

(11) H. Peters, U. S. Pat. 1,008,557 (1911).

(12) M. Hagedorn and P. Miller, *Cellulosechem.* **12**, 29 (1931).

(13) J. F. Haskins and D. C. Ellsworth, U. S. Pat. 2,102,205 (1937).

(14) H. Dreyfus, U. S. Pat. 1,502,379 (1924).

(15) H. Dreyfus, Brit. Pat. 346,806 (1929).

(16) L. Lilienfeld, Brit. Pat. 149,318 (1920).

cellulose, which is now in the form of "crumbs," is placed in an autoclave, the etherifying agent is added and the mixture is heated, with stirring, to a suitable temperature. Purification is accomplished in various ways depending on the nature of the product. Many modifications of this general procedure have been proposed. Since these modifications are designed to overcome one or more of the difficulties attendant on the process, they will be discussed after a brief study of the chemistry of the reaction.

## V. CHEMISTRY OF THE ETHERIFICATION

### 1. *Influence of the Colloidal Nature of the Cellulose*

Cellulose is a hydrophilic colloid. It is insoluble in aqueous alkali under ordinary conditions but is highly swollen by it. The extent of the swelling varies with the previous history of the cotton or wood pulp being used. The "swelling factor" of the linters or wood pulp has long been recognized as an important factor in the production of viscose since a high swelling factor usually represents a high degree of reactivity while a low swelling factor enables the manufacturer to put more pulp in his presses, thereby increasing production.

According to our modern viewpoint, cellulose is composed of long chain-like molecules which are oriented in the plant in a parallel manner, although there appear to be amorphous areas even in native cellulose. The molecules are held together to form "micells" by powerful secondary forces. These forces have been measured and found to be of a magnitude consistent with the presence of hydrogen bonding.<sup>17</sup> Not only are the molecules themselves arranged in this way but the structure of the plant is such that fibers, cells, and growth rings are present. All this brings about a situation such that the etherifying agent, in order to penetrate to the center of the fibers or micells and so reach the hydroxyl groups present, must diffuse through or between the fibers against the forces of the secondary valences which hold the molecules together.

### 2. *Influence of the Nature of the Hydroxyl Groups of the Cellulose*

Two of the hydroxyl groups present in each glucose unit of the cellulose are secondary, one is primary. Studies on the relative rates of etherification of the three hydroxyls are not conclusive. Mahoney and Purves,<sup>18</sup> using a method which takes advantage of the rapid reaction between tosyl chloride and a primary hydroxyl, combined with a determination of unsubstituted glycol groups, have studied the relative num-

(17) H. Mark, *Chem. Revs.*, **26**, 169 (1940).

(18) J. F. Mahoney and C. B. Purves, *J. Am. Chem. Soc.*, **64**, 19 (1942).

bers of primary and secondary alcohol groups remaining unetherified in partially ethylated and methylated cellulose and obtained data which indicate that the rate of etherification of the primary hydroxyl is approximately twice that of one of the secondary hydroxyls. Spurlin<sup>19</sup> has discussed the distribution of substituent groups in a partially substituted cellulose, starting with the assumption that all of the hydroxyl groups of the cellulose are equally exposed to the etherifying agent and that the ratios of the rates of reaction of the primary and secondary hydroxyls are 10:1:1 or 1:1:1. According to this study the effect of varying reactivities on the overall distribution is not very great. In any case it would appear that other conditions have much more influence on the distribution of the substituent groups than the relative reactivities of the three hydroxyl groups.

The hydroxyl groups of the cellulose appear to be somewhat acidic. While studies of the composition of alkali cellulose and adsorption of sodium hydroxide have not clearly proved the presence of any sodium compound in alkali cellulose, the reactions of alkali cellulose with carbon disulfide and with etherifying agents would seem to justify the assumption that such an intermediate exists or that the hydroxyl hydrogen at least ionizes. This view is strengthened by the fact that the rate of etherification is proportional to a high power of the concentration of alkali.<sup>19</sup>

### 3. *Effect of Alkali Concentration*

The concentration of alkali in the alkali cellulose is important for three reasons; first, because it influences the degree of swelling. This, of course, affects the ease of penetration of the etherifying agent and therefore the uniformity of etherification. The greatest swelling occurs at about 14% sodium hydroxide concentration but this optimum swelling concentration varies with the temperature, swelling being generally greater at low temperatures, while the optimum concentration of alkali is lower at the lower temperatures. Second, as has been stated, the rate of etherification increases rapidly with the concentration of alkali. A third consideration is that, as etherification proceeds, alkali is consumed not only in the etherification but also in side reactions. Both of these latter considerations dictate a high concentration of alkali in the reaction mixture and it is necessary to sacrifice some of the swelling effect of the lower concentrations in order to gain the driving force of the higher alkali concentration.

(19) H. M. Spurlin, *J. Am. Chem. Soc.*, **61**, 2222 (1939).

#### 4. *Influence of the Nature of the Etherifying Agent*

As stated earlier, the chemical nature of the reagent is in many respects overshadowed by its physical nature. Solubility of the reagent in the swelling solution, the rate of diffusion into the cellulose, and the rate of saponification of the reagent all have an important bearing on its efficiency. Relatively soluble compounds such as sodium chloroacetate and relatively mobile reagents such as methyl and ethyl chlorides and ethylene oxide will give a more uniform etherification and a lower loss by side reactions. In the experience of the writer, bifunctional reagents such as ethylene bromide or  $\beta,\beta'$ -dichlorodiethyl ether give only a low degree of substitution and the products are highly insoluble and are only slightly swollen in alkali. This points to cross-linking with the resultant formation of materials of extremely high molecular weight. This cross-linking effect of polyfunctional reagents is so marked in cellulose reactions generally that care should be taken to insure the absence of even traces of such materials. The presence of a little dichlorohydrin or epichlorohydrin in glycerol monochlorohydrin, for example, might render the latter useless for the production of a soluble cellulose ether.

#### 5. *Changes Occurring during the Reaction*

During the progress of an etherification, as for example with benzyl chloride, the hydrophilic properties of the cellulose are lost. It becomes less swollen by the aqueous alkali and more swollen by the organic reagent. Consequently the cellulose shrinks as it is etherified, squeezing out the alkali and absorbing more of the alkylating agent. In the last stages of the etherification, therefore, the difficulty lies in the inaccessibility of the remaining hydroxyls to the alkali rather than to the alkylating agent. The course of such a reaction has been followed microscopically by Lorand and Georgi with very illuminating results.<sup>5</sup>

### VI. MODIFICATIONS OF THE PROCEDURE

The above-presented picture of the etherification reaction suggests certain modifications, each designed to overcome one or more of the technical difficulties which attend such reactions.

#### 1. *Steps to Secure Greater Swelling or Dispersion of the Cellulose*

*a. Use of Regenerated Cellulose.* Regenerated cellulose, and particularly regenerated cellulose of a low degree of polymerization, is relatively soluble in alkali. A dispersion of such material in alkali should, for this reason, be more readily accessible to an etherifying agent than fibrous

cellulose. The use of regenerated cellulose as a raw material for etherification has been proposed. The products would have a low viscosity.

*b. Sodium Cupri-cellulose.* If cellulose is impregnated with a copper salt and is then treated with 15–16% aqueous sodium hydroxide, a deep blue, highly swollen complex is formed. When treated with dimethyl sulfate under suitable conditions, this complex gives a distribution of methyl groups closely approaching the theoretical.<sup>20–25</sup>

*c. Dispersion in Quaternary Ammonium Bases.* Cellulose is soluble in or is very highly swollen by aqueous solutions of certain quaternary ammonium bases of high molecular weight. Such dispersions have been proposed as starting materials for cellulose etherifications.<sup>26–28</sup> While it is probably true that the etherification will be more uniform in its early stages when such dispersions are used it has also been shown that these are not true molecular dispersions and that the over-all etherification is not entirely uniform.<sup>28</sup>

*d. Simultaneous Deacetylation of Cellulose Acetate and Etherification.* By treating an acetone solution of cellulose acetate with alkali and an etherifying agent simultaneously, deacetylation takes place with the production of regenerated cellulose in a finely divided form readily accessible to the etherifying agent. By this method, which has been shown to give a relatively uniform substitution, Haworth<sup>29</sup> was able to obtain a completely methylated cellulose in one step. This method is of theoretical interest but suffers from the practical objections that it would be expensive commercially and the cellulose obtained by deacetylating cellulose acetate would possess a very low degree of polymerization. All of these methods of obtaining better dispersion of the cellulose are fairly satisfactory when dealing with methylcellulose or hydroxyethylcellulose since these products are water-soluble. With ethylcellulose or benzylcellulose or other ethers which become more hydrophobic in character as the degree of substitution increases, the effect of a good original dispersion or swelling is soon lost, the cellulose starts shrinking in the alkali, even squeezing the latter out, so that, in the later stages of the etherification the reaction may stop because the alkali is not available.<sup>5</sup>

(20) W. Traube and A. Funk, *Ber.*, **69 B**, 1476 (1936).

(21) W. Traube, R. Piwonka and A. Funk, *Ber.*, **69 B**, 1483 (1936).

(22) T. Lieser, *Ann.*, **528**, 276 (1937).

(23) R. Piwonka, *Ber.*, **69 B**, 1965 (1936).

(24) W. A. Sisson and W. R. Sauer, *J. Phys. Chem.*, **43**, 687 (1939).

(25) W. J. Heddle and E. J. Percival, *J. Chem. Soc.*, 249 (1939).

(26) L. H. Bock, *Ind. Eng. Chem.*, **29**, 985 (1937).

(27) T. Brownsett and D. A. Clibbens, *J. Textile Inst.*, **32**, T32 (1941).

(28) J. Compton, *J. Am. Chem. Soc.*, **60**, 2823 (1938).

(29) W. N. Haworth, E. L. Hirst and H. A. Thomas, *J. Chem. Soc.*, 821 (1931).

## 2. Steps to Maintain High Alkali:Cellulose Ratio

In order to minimize the changes in concentration of alkali as etherification proceeds, a high original concentration of alkali is advisable. It has also been proposed to add alkali as the reaction progresses or to add alkali and etherifying agent simultaneously at successive stages.<sup>30</sup> Etherification in steps with isolation of the intermediate has also been proposed. This has several advantages. First, it permits removal of large quantities of salt formed in the first stages. Second, the partially substituted cellulose obtained in the first step is more highly swollen in the alkali or, at a higher degree of substitution, will more readily absorb the alkylating agent. The latter consideration may be of importance in making mixed ethers.<sup>13</sup>

## 3. Etherification in Stages

As cellulose is etherified, for example by ethyl chloride, a progressive change takes place in the properties. At first the partially ethylated cellulose becomes even more highly swollen in alkali than the original cellulose. As ethylation proceeds this trend reverses and the ethylated cellulose changes progressively from a highly hydrophilic colloid to a hydrophobic one, no longer swollen by alkali but increasingly swollen in and ultimately soluble in organic solvents (see description of benzyla-tion). After the introduction of about one equivalent of ethoxyl group per glucose unit of the cellulose, the ether becomes sufficiently swollen by organic solvents to absorb them preferentially. This permits etherification of the remaining hydroxyl groups by higher alkyl halides, such as butyl, amyl, "lauryl," and "stearyl," which do not react well with the unsubstituted cellulose.<sup>13</sup> These mixed ethers, because of the greater molecular weight of the higher alkyl groups, have the properties which would be expected of the higher ethers.

## 4. Control of Viscosity

The physical properties of films or filaments of cellulose ethers, such as tensile strength, flexibility, toughness, softening point and solubility are closely related to the degree of polymerization of the product. In this respect both the average degree of polymerization and the uniformity of chain length are of significance. It is usually necessary to compromise somewhat in choice of viscosity since, while good physical properties are associated with high molecular weight, a certain degree of degradation is necessary in order to obtain solutions which are not too viscous to handle

at desired concentrations. Control of viscosity is, therefore, very important in the manufacture of cellulose ethers.

*a. Methods of Viscosity Reduction.* Cellulose ethers of medium or low viscosity may be obtained by several methods.

(1) *Use of low viscosity cellulose.* Cellulose which has been degraded by acids or by other means may be used as a starting material. Hydrocellulose and cellulose regenerated from viscose have been used.

(2) *Aging of alkali cellulose.* In the preparation of alkali cellulose, an important step is the aging of the shredded alkali cellulose crumbs. This process is essentially a degradative oxidation brought about by the action of atmospheric oxygen in the presence of alkali. To date this method has given the most uniform viscosity reduction. It can be controlled easily by regulating the time of aging. A complete discussion may be found elsewhere. It will suffice at this point to say that this process appears to afford the best method of viscosity control.

(3) *Action of acids on cellulose ethers.* Cellulose ethers, like cellulose, are hydrolyzable by acids. Methods for viscosity reduction of the ethers have been proposed which involve treatment of the ether with sulfuric acid<sup>31</sup> or with gaseous hydrogen chloride.<sup>32</sup>

*b. Methods of Maintaining Viscosity.* It has been shown that degradation of cellulose in the presence of alkali is due to oxidation. Consequently a considerable degradation takes place during etherification if air is present. This may be minimized by etherifying under nitrogen. In order to avoid as much oxidative degradation as possible it would probably be necessary not only to carry out the etherification in the absence of air but to steep, press and shred the cellulose in the absence of air. Addition of phenols and other antioxidants to the alkali has also been proposed.

## VII. ETHERS OF CELLULOSE SOLUBLE IN ORGANIC SOLVENTS

For use as a plastics material, in lacquers, or for the preparation of water-insensitive films or filaments, ethylcellulose of a relatively high degree of substitution (1.8–2.8) and the aralkyl ether, benzylcellulose (degree of substitution 1.4–2.5) have had the greatest commercial development.

### 1. *Organo-soluble Ethylcellulose*

Ethylcellulose is made commercially by the action of ethyl chloride on an alkali cellulose prepared by steeping cotton linters or wood pulp

(31) A. Ascherl and W. Gruber, U. S. Pat. 1,834,040 (1934).

(32) E. Reid, U. S. Pat. 1,864,554 (1932).

in caustic soda of about 50% concentration. Optimum conditions are claimed to be steeping in 50% sodium hydroxide and treating with six moles of ethyl chloride to one of cellulose for eighteen hours at 120–130°C.<sup>33</sup> The reaction requires an elevated temperature and must be carried out under pressure. Purification of the product is comparatively simple since it can be washed free of by-products by a water and acid treatment. Solvents for the product, such as benzene, may be present during the reaction and may be steam-distilled from the reaction mixture and recovered.<sup>3,34</sup>

*a. Properties.* The properties of ethylcellulose depend so much upon the degree of polymerization as well as upon the degree of uniformity of substitution that no accurate tabulation of properties can be made. Manufacturers' bulletins<sup>35,36</sup> should be consulted for such information. Some generalizations can, however, be made.

(1) *Solubility.* An increase in the degree of substitution of ethylcellulose, since this progressively changes the nature of the product from the original hydrophilic nature of cellulose to a decidedly hydrophobic nature, increases the solubility in hydrocarbons.<sup>37</sup> Solubility in alcohols at first increases with degree of substitution and then decreases.<sup>37</sup> One of the interesting properties of the cellulose ethers is their solubility in mixed solvents such as alcohol-benzene. In some cases an ether can be prepared which is not soluble in either of two solvents but will dissolve in a given mixture of the two. The degree of substitution which gives maximum solubility will vary with the composition of the mixed solvent.

(2) *Softening point.* The softening point of ethylcellulose varies with the degree of substitution as well as with the degree of polymerization. This effect parallels the solubility and the maximum solubility in mixed solvents coincides fairly closely with the lowest softening temperature.<sup>37</sup> The softening temperature of ethylcellulose is lower than that of cellulose acetate and this is the chief reason that it is not as suitable for rayon manufacture. It may be pointed out that the softening points of the higher ethers such as the butyl or amyl, are still lower than that of the ethyl ether. The degree of polymerization of the ether has a marked influence on the softening point, a low specific viscosity being associated with a low softening point.<sup>37</sup>

(33) S. N. Uschakov and I. Schneer, *Plasticheskie Massy*, No. 1–2, 17 (1931); *C. A.*, **26**, 3373 (1932).

(34) F. C. Hahn, U. S. Pat. 1,819,600 (1931).

(35) Ethocel, Dow Chemical Co., Midland, Michigan.

(36) Ethyl Cellulose, Hercules Powder Co., Wilmington, Delaware.

(37) E. J. Lorand, *Ind. Eng. Chem.*, **30**, 527 (1938).



(3) *Tensile strength.* Since the tensile strength of cellulose derivatives is associated with the secondary forces which act between the cellulose chains, a high degree of substitution might be expected to be associated with a low tensile strength. This appears to be the case. Even more important is the average degree of polymerization and the uniformity of molecular size. The presence of any considerable quantity of material of a low degree of polymerization, as shown by fractionation experiments, will result in a low tensile strength and flexibility.<sup>38</sup> In general the softening point of the ether can be taken as a good indication of the physical properties of films made from the ether.

*b. Uses.* Organo-soluble ethylcellulose is being manufactured in considerable quantities in the United States for use in the manufacture of plastics, lacquers and films. Its high compatibility with solvents, particularly cheap mixed solvents, its relative nonflammability, resistance to hydrolysis, and the excellent properties of films made from it render it suitable for films and coatings. Its relatively low softening point and compatibility with plasticizers and softeners adapt it for use in plastics, particularly in injection molding.

## 2. Benzylcellulose

*a. Preparation and Purification.* The benzyl ether of cellulose is prepared by the action of benzyl chloride on cellulose in the presence of alkali.<sup>39,40</sup> The reaction proceeds quite well but considerable difficulty is encountered in purification due to the difficulty of removing benzyl alcohol, dibenzyl ether and excess benzyl chloride from the product. Methods for purification include: (1) successive extraction with water and alcohol<sup>41</sup>; (2) steam distillation to remove by-products<sup>41</sup>; (3) mixing with a water-soluble salt, in order to break up the gummy reaction mass, followed by extraction with alcohol<sup>42</sup> or steam-distillation.<sup>43</sup> The course of the benzylation has been followed microscopically by Lorand and Georgi.<sup>5</sup>

Benzylcellulose has been developed in Europe more extensively than in the United States. Depending upon the degree of substitution, the degree of polymerization, uniformity of substitution and other factors, benzylcellulose preparations may be obtained which are compatible with

(38) S. N. Ushakov, I. M. Geller and E. M. Demina, *Plasticheski Massy, Sbornik Statei*, 58 (1939); *Khim. Referat. Zhur.*, No. 3, 102 (1940); *C. A.*, 36, 3043 (1942).

(39) M. Gomberg and C. Buchler, *J. Am. Chem. Soc.*, 43, 1904 (1921).

(40) D. Thrall, *Brit. Pat.* 327,714 (1930).

(41) L. Lilienfeld, *U. S. Pat.* 1,858,019 (1932).

(42) E. Doerr, *U. S. Pat.* 1,771,529 (1930).

(43) G. Okada, *Jap. Pat.* 93,058 (1931); *C. A.*, 26, 4470 (1932).

a variety of plasticizers and which are soluble in a wide variety of solvents such as benzene-alcohol, acetone-benzene, chloroform or cyclohexanol. Benzylcellulose is relatively non-hygroscopic, the moisture content varying from 0.5% to 1% and the hygroscopicity decreasing with degree of substitution. It is unaffected by alkali. Benzylcellulose is thermoplastic at 110°C. and is quite satisfactory for molding purposes. It has been suggested for use in many fields such as insulation, lacquers, floor coverings, dentures, fabrics, and filtering materials. Its chief disadvantage is very probably its relatively low softening temperature.

### VIII. WATER-SOLUBLE ETHERS OF CELLULOSE

The introduction of certain types of ether groups into the cellulose molecule enhances the hydrophilic properties of the latter. In particular, such groups as methoxyl, hydroxyethyl and carboxymethyl, when present in the proper amounts, render the cellulose derivative soluble in water.

#### 1. *Methylcellulose*

The solubility of methylcellulose in water is dependent upon: (1) the degree of substitution; (2) the degree of polymerization; (3) the temperature of the water; and (4) the uniformity of substitution. Consequently no definite limits can be stated. Certain generalizations can, however, be made.

*a. Effect of Degree of Substitution on Solubility.* With increasing degree of substitution, methylcellulose of a given degree of polymerization becomes increasingly swollen by water until at a degree of substitution of about 0.7–2.6 a colloidal solution can be obtained in cold water. At higher degrees of substitution the solubility in water again decreases.

*b. Effect of Degree of Polymerization on Solubility.* As is the case with all cellulose ethers, solubility of methylcellulose in water is improved as the degree of polymerization is decreased.

*c. Effect of Temperature on Solubility.* Methylcellulose tends to become less soluble as the temperature of the solution rises and to increase in solubility as the temperature falls. A solution of methylcellulose, prepared by cooling, forms a thixotropic gel as the temperature is raised.<sup>44</sup> This gel can be coagulated at relatively high temperatures. This interesting property permits easy purification of the reaction product. The exact temperature of gelation will depend on the other factors involved in solubility.

(44) E. Heymann, *Trans. Faraday Soc.*, **31**, 846 (1935); **32**, 462 (1936).

*d. Effect of Uniformity of Substitution on Solubility.* Methylcellulose of a degree of substitution of about 0.7 will be soluble in water provided it has been prepared by a method which gives uniform substitution, as for example, by the action of methyl chloride on sodium cupricellulose. If the substitution is not uniform such a methylcellulose will not give good solutions.

*e. Preparation of Methylcellulose.* Water-soluble methylcellulose can be prepared by a one step etherification provided that the ratio of alkali to water in the alkali cellulose is maintained at a high level.<sup>45</sup> While methyl sulfate and methyl chloride have both been used as etherifying agents, the latter provides a more uniform etherification and is thus preferable.<sup>46</sup> Purification is accomplished by washing the reaction product in hot water.

## 2. Water-soluble Hydroxyethylcellulose (Glycol Cellulose)

When alkali cellulose is treated with ethylene chlorohydrin or ethylene oxide, hydroxyethyl groups are introduced into the cellulose molecule.<sup>47</sup> While the reaction of chlorohydrin may be considered as analogous to the action of ethyl chloride it is more probable that it reacts through the intermediate formation of ethylene oxide. The latter reacts by addition at the hydroxyl groups to give hydroxyethyl groups. The introduction of hydroxyethyl groups does not change the number of hydroxyl groups in the molecule so that, as etherification proceeds, the ether becomes more and more hydrophilic and a water-soluble product results at a degree of substitution of 1.5 to 2.5.

The water-soluble hydroxyethylcellulose has only a limited use as compared to water-soluble methylcellulose, principally because of the difficulty of isolating the product. Aqueous solutions of hydroxyethylcellulose do not gel on heating and the product is about as soluble in hot water as in cold, so that the method used for isolating water-soluble methylcellulose is not applicable. However, by dialyzing the reaction product the alkali and salts may be removed and the product isolated.

The properties of hydroxyethylcellulose are like those of methylcellulose except for the fact that there is little or no temperature effect on solubility. The degree of substitution required to impart water solubility will depend both upon the degree of polymerization of the cellulose and upon the uniformity of substitution. It is of interest

(45) K. Hess, G. Abel, W. Schon and V. I. Komarewsky, *Cellulosechem.*, **16**, 69 (1935).

(46) A. Maasberg, U. S. Pat. 2,160,782 (1939).

(47) A. W. Schorger and M. J. Shoemaker, *Ind. Eng. Chem.*, **29**, 114 (1937).

that the hydroxyl group of the hydroxyethyl radical is itself capable of etherification and it may be supposed that the more highly etherified products will contain some chains of repeated glycol ether groups.

### 3. *Water-soluble Carboxymethylcellulose (Cellulose Glycolic Acid)*

Treatment of alkali cellulose with sodium chloroacetate results in an ether with a free carboxyl group. This ether, in the form of its sodium salt, is water-soluble even when the degree of substitution is relatively low. Since the alkali-soluble modification of this substance is of much greater industrial importance it will be discussed in detail under that heading.

## IX. CELLULOSE ETHERS SOLUBLE IN DILUTE ALKALI BUT NOT IN WATER

Cellulose is much more highly swollen in alkali than in water. When only a slight amount of an ether group such as methyl, ethyl, hydroxyethyl or carboxymethyl is introduced along the cellulose chain, the swelling tendency is greatly increased. It may be assumed that this disproportionate effect is caused by the separation of the cellulose chains produced by these ether groups and the resulting reduction of the secondary valences which hold them together. When this effect is associated with the fact that cooling enhances the swelling effect of the alkali it may be seen that a great variety of ethers of a low degree of substitution may be obtained. Thus there have been described cellulose ethers that are soluble in alkali at room temperature and others that are soluble in alkali only below room temperature.<sup>48,49</sup> The degree of substitution required to give a product soluble in alkali will depend upon the temperature of the solution as well as upon the uniformity of substitution and the degree of polymerization. Ethers of about 0.5 mole of substitution or less are the most important. Because of the possibility of obtaining a soluble cellulose derivative with only a slight degree of substitution, coupled with the possibility of regenerating the derivative by an acid treatment, these alkali-soluble ethers have been the subject of a great deal of industrial research.

### 1. *Methyl- and Ethylcellulose*

By introduction into cellulose of about 0.5 equivalent of methyl or ethyl groups a product is readily obtained which can be dissolved in alkali of 4–10%. The ether may be regenerated from this solution by

(48) L. Lilienfeld, U. S. Pat. 1,589,606 (1926).

(49) D. C. Ellsworth and F. C. Hahn, U. S. Pat. 2,157,530 (1939).

neutralizing the alkali, following much the same procedure as that followed in regenerating cellulose from viscose. Use of such solutions in making threads or films is possible but is rendered difficult by the fact that the threads or films are much more sensitive to water than regenerated cellulose. Such products also tend to give horny products on drying that are difficult to handle.

### 2. *Hydroxyethylcellulose*

The low-substituted hydroxyethylcellulose which, like methyl- and ethylcellulose, is soluble in alkali, particularly when cooled, has much to recommend it from an industrial point of view. It can be formed by the action of only small quantities (0.25 to 0.5 moles) of ethylene oxide on alkali cellulose.<sup>47</sup> The reaction product need not be isolated since there are no salts formed, but may be diluted with water or weak alkali to give a spinning solution. The product should therefore be quite cheap. Preparation and properties of hydroxyethylcellulose have been discussed by Schorger and Shoemaker.<sup>47</sup>

### 3. *Carboxymethylcellulose*

By the action of relatively small quantities of sodium chloroacetate on alkali cellulose, carboxymethyl ethers are obtained which give smooth solution in dilute alkali but which can be regenerated to give threads or films of high tensile strength. These products are relatively hygroscopic. The substances are soluble in the form of their sodium salts and form insoluble salts with many metals.<sup>8</sup>

## X. USE OF CELLULOSE ETHERS AS INTERMEDIATES

As has been shown, one of the difficulties in making derivatives of cellulose is the difficulty in obtaining solutions of cellulose that give uniform reactions. Partial substitution of the cellulose by suitable groups gives an intermediate that is soluble in alkali and that is subject to such reactions as can be carried out in the presence of alkali. Several reactions of this type have been suggested. Alkaline solutions of such ethers are suitable for the Schotten-Baumann reaction, for example, to prepare benzoic or sulfonic acid ether-esters of cellulose.<sup>50</sup> Hydroxyethyl- and carboxymethylcelluloses are of interest also because of the reactive groups in the side chain. Ethers of a somewhat higher degree of substitution may be esterified by the usual methods to give cellulose ether-esters, such as ethylcellulose acetate. Many of these have been described but have as yet led to no important commercial developments.

(50) J. F. Haskins, U. S. Pat. 2,146,738 (1939).

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## ERRATA

### VOLUME 1

Page 24, line 3 under section 4. For "z-propanol" read "2-propanol."

[ Page 25, footnote 73. For "Diehls" read "Diels."

Page 35, line 3 from bottom. For "D-Manno-L-manno-octitol" read "Manno-manno-octitol" since the structure is meso.

Page 59, last line. For "trihydroxypyrazole" read "5-(D-erythro-1,2,3-trihydroxypropyl)pyrazole."

Page 111, line 2 below formula. Reference number 56 should be 5, 6.

Page 181. The D and L in the two sorbitol formulas should be reversed.

Page 231, formula XI. Insert bond between P and OH at bottom.

Page 275, line 13. For "hydroxymethyl" read "hydroxyethyl."

Page 276, line 10 from bottom. For "carbonyl" read "carboxyl."



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